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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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FURTHER OBSERVATIONS ON THE PHENOMENA ENCOUNTERED IN ATTEMPTING TO TRANSMIT VARICELLA TO RABBITS.

By THOMAS M. RIVERS, M.D., and WILLIAM S. TILLETT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATE 38.

(Received for publication, February 18, 1924.)

The phenomena observed following the injection of blood from varicella patients into the testicles of rabbits and the macroscopic lesions resulting from the inoculation of testicular emulsions from these rabbits into the testicles, cornea, and skin of other rabbits in series have been described in a preceding paper.[1] At the time of the previous report, however, the results of inoculations of the skin with active testicular emulsions were so variable that it was impossible to predict regularly whether a visible reaction would occur in any individual rabbit even when a concentrated virus was smeared on the scarified skin. This irregularity in the occurrence of the skin reactions interfered with rapid progress in the study of the phenomena observed. Therefore a better method of inoculating the skin was sought, and the intradermal injections of small quantities of the active testicular emulsions were soon found to produce constant and specific reactions in the skin of rabbits. A description of the intradermal method of inoculation and the results of the experimental work facilitated by its use will be given in this paper.

Intradermal Inoculation.

Method.

Testicles removed[2] from rabbits 4 days following intratesticular injections of virus, were ground up with sterile, chemically clean sand and 10 cc. of Locke's solution. The testicular emulsion was centrifuged for 10 minutes at high speed. Por-

[1] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673.

[2] All operations were performed under ether anesthesia.

tions of the decanted supernatant fluid were cultured on various media for ordinary anaerobic and aerobic bacteria. With other portions of the supernatant fluid and Locke's solution a series of dilutions from 1:1 to 1:1,000 was made and 0.2 cc. of each dilution was injected by means of a tuberculin syringe into the shaved skin of a rabbit. White elevated areas in the skin about 1 cm. in diameter appeared immediately at the sites of the intradermal injections and then rapidly disappeared. Daily examinations of the skin were made for 2 weeks.

Following the intradermal injections nothing except the effects of trauma was observed until the 3rd or 4th day, when it was noticed that the skin at the sites of the inoculations of concentrated material was red and raised (Fig. 1). The reactions at these points increased in size and intensity till the 5th to 7th day when the central portion of the inoculated areas was often very dark. Extending into the skin around the dark central area was a zone of less intense erythema 2 to 4 cm. in width. The lesions gradually subsided and in a fortnight disappeared without scar formation. At the sites of inoculation of the higher dilutions of the virus, visible reactions appeared more slowly,—5th or 6th day,—and they were smaller, less severe, and disappeared more rapidly.

In developing the method of intradermal inoculations of the virus, adequate controls were conducted by injecting fresh and heated emulsions of normal testicles into the skin of normal rabbits and fresh testicular emulsion containing active virus into the skin of immune rabbits. No reactions were seen in the controls. The absence of reactions following the control injections was so striking that it soon became evident that the skin lesions following the injections of testicular emulsions containing active virus were specific. While the method of smearing the testicular emulsions on the scarified skin was adequate for demonstrating the presence of a virus (Fig. 2); the results were so variable that it was impossible to titrate the virus accurately, to determine the effects of certain physical and chemical agents upon the virus, or to complete immunological studies with the virus. The results obtained following the introduction of the intradermal method, however, were so consistent and reliable as to facilitate the completion of the desired experiments in a satisfactory manner.

Virus III.

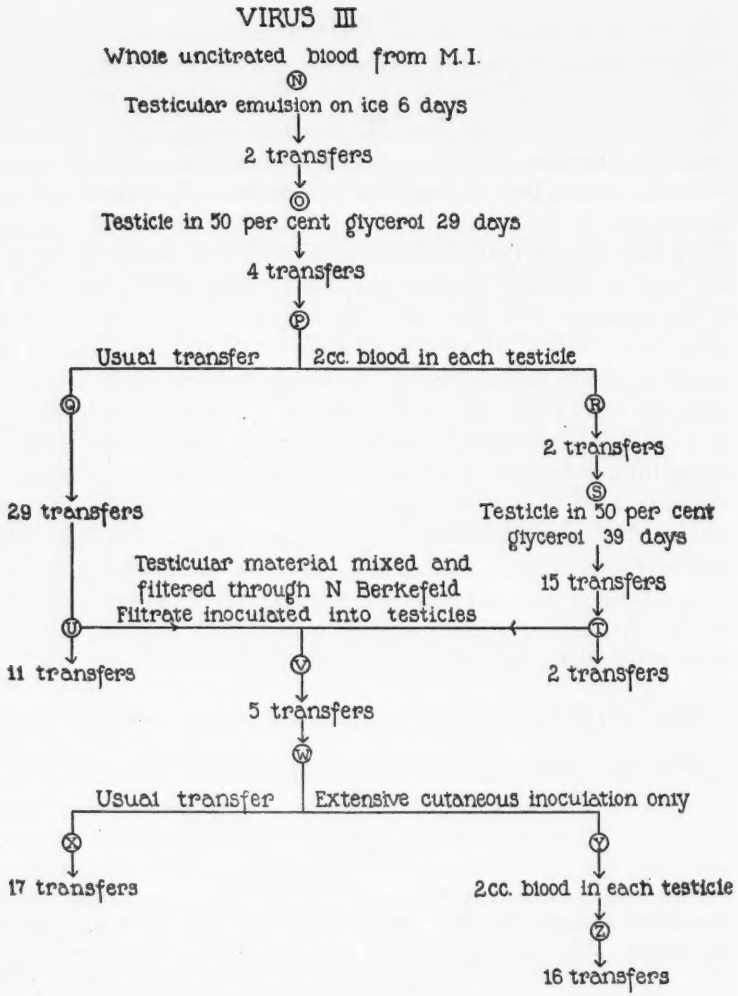
Before proceeding with a detailed description of the experiments, a brief statement concerning the virus used in most of them will be made. The lesions produced by Virus III were first recognized in the fourth testicular transfer following a primary inoculation of blood from a varicella patient into the testicles of a rabbit.[1] The virus has been transferred 65 times from rabbit to rabbit (Text-fig. 1). It now acts like a fixed virus in that a small amount of the virus when injected into the testicles of rabbits causes a sharp rise of temperature to 104–106°F. in the animals 3 or 4 days after the inoculation, and because intradermal injections of the virus in dilutions of 1:1,000 are regularly followed by visible skin reactions. Although the virus can be recovered from the heart's blood of rabbits (Text-fig. 1) following intratesticular or intradermal inoculations, it does not kill the animals even when injected intracerebrally. Portions of the testicular emulsions containing the virus have been shown repeatedly to be free of ordinary anaerobic and aerobic bacteria by means of cultures on blood agar, in broth, and in Smith-Noguchi tubes. Furthermore, ordinary bacteria have not been seen in stained films and dark-field preparations of the emulsions containing the virus, in stained sections of inoculated testicles, or in sections of inoculated testicles impregnated with silver nitrate.

Effects of Certain Physical and Chemical Agents upon the Virus.

Filtration.—Virus III passes through Berkefeld filters N and V (Table I). Filters W were not used.

Method.

Fresh testicular emulsions were prepared in the usual manner except that at times larger amounts, 20 to 40 cc., of Locke's solution were added to the ground up testicles. After the emulsions had been centrifuged at high speed for 10 minutes, the supernatant fluid was decanted and filtered through paper. Influenza bacilli or colon bacilli, occasionally both, were added to the emulsions before filtration was started. This was done in each experiment to test the filters simultaneously with the filtration of the virus. Some of the filters were new, while others had been used two or three times previously. A water suction apparatus created the vacuum necessary for the filtration in all the experiments except one in which the filtration was accomplished under 50 pounds of pressure. The time



Transfers = consecutive transfers from the testicles of one rabbit to those of another.

Usual transfer = usual testicular transfers.

TEXT-FIG. 1. Outline of the transfer method pursued in the study of Virus III.

required to obtain enough filtrate for the experiments varied from 10 to 60 minutes. Portions of each filtrate were cultured aerobically and anaerobically in liquid and on solid media with and without blood. Other portions of the filtrate were injected into the skin and testicles of normal rabbits and into the skin of immune rabbits. In many of the experiments, testicles which had been inoculated with the filtrates were removed 4 days later and ground up in the usual manner. Then portions of these emulsions were injected into the skin and testicles of normal rabbits. In this way the virus in the filtrates was passed through several rabbits by means of testicular inoculations.

The cultures of the filtrates in the nine experiments summarized in Table I remained free of ordinary bacteria. In the first three experiments no visible reactions occurred in the rabbits' skin at the sites of inoculation of the filtrates. The animals in two of these experiments were immune, however, when they were inoculated intradermally with active virus 2 weeks later. In the other six experiments, intradermal inoculations of the filtrates produced visible reactions in the skin of normal rabbits but none in the skin of immune rabbits. Testicular emulsions from Rabbits 11 and 12 were mixed and filtered. Portions of the filtrate were injected into the skin and testicles of Rabbit 13. The virus from this filtrate has been passed through twenty-four rabbits by means of testicular inoculations and is now being carried as a stock strain. Rabbits 11, 12, and 13 in Table I are Rabbits T, U, and V in Text-fig. 1.

In the 8th and 9th experiments (Table I), the testicular emulsions before and after filtration were titrated in the skin of the same rabbit. In the 8th experiment the emulsion before it was filtered produced a visible reaction in the skin when diluted 1:1,000, yet the same emulsion after it was filtered produced such a reaction only when undiluted. In the 9th experiment the unfiltered emulsion diluted 1:200 produced a visible skin lesion, while the filtered emulsion produced no such lesion in dilutions higher than 1:10. Some of the virus passed through the filters, yet it is obvious that most of it was held back under the conditions of the experiments. No attempt will be made to discuss all the factors which may influence filtration. It seems, however, that filters which have been used several times are more impervious than unused ones, and that a relatively greater concentration of the virus passes through the filters when the testicular emulsions have been diluted. These two factors may account for the results of the titrations of the

TABLE I.

Summary of the Results of Nine Experiments Showing Conclusively that Virus III Passes through Berkefeld Filters N and V.

Testicular virus from rabbits	Berkefeld filter	Suction or pressure	Time of filtration	Test bacteria	Sterility of filtrate	Filtrate inoculated intradermally and intratesticularly into rabbits	Result of skin inoculations	Immune after 2 weeks
1	N	Water pump	10 min.	Influenza bacillus	Sterile	2	−	Yes
						↓		
						3	−	Yes
4	N	Water pump	15 min.	Influenza bacillus	Sterile	5	−	No
						↓		
						6	−	No
						↓		
						7	−	No
8	N	Water pump	30 min.	Influenza bacillus	Sterile	9	−	Yes
"	"	"	"	"	"	10	−	Yes
11 (T) and 12 (U)	N	Water pump	30 min.	Influenza bacillus	Sterile	13 (V) ↓ Still being passed from rabbit to rabbit by testicular inoculations	+	Yes
14	N	50 pounds pressure	30 min.	Influenza bacillus	Sterile	15	+	Yes
						↓		
						16	+	Yes
						↓		
						17	+	Yes
						↓		
						18	+	Yes
19	V	Water pump	10 min.	Influenza bacillus	Sterile	20	+	No test
						↓		
						21	+	No test
22	V	Water pump	60 min.	Influenza bacillus	Sterile	23	+	Yes
24	V	Water pump	60 min.	Colon bacillus	Sterile	25	+	Yes
26	V	Water pump	10 min.	Colon bacillus and Influenza bacillus	Sterile	27	+	No test
						↓		
						28	+	Yes

↓ = Passage of the virus from one rabbit to another by means of testicular inoculations.

Intradermal inoculations were used in testing for the presence of an immunity.

All intradermal inoculations were controlled with normal and with immune rabbits.

− = No visible skin reaction; + = visible skin reaction.

testicular emulsions before and after filtration in the 8th and 9th experiments. In the former the filter had been used twice previously and the testicular emulsion was concentrated, while in the latter the filter had not been used previously and the emulsion was diluted before filtration was attempted.

In the above experiments it has been shown conclusively that Virus III is filterable in spite of the fact that a great deal of it is held back by the filters.

TABLE II.

Summary of Results of Two Experiments Showing the Effect of Heat upon Virus III.

Testicular virus from rabbit	Test rabbit	Unheated virus control	Heated virus						
			30 min. 45°C.	30 min. 55°C.	30 min. 65°C.	10 min. 55°C.	20 min. 55°C.	40 min. 55°C.	50 min. 55°C.
1	2 normal	+	+	−	−	−	−	−	−
1	3 immune	−	−	−	−	−	−	−	−
4	5 normal	+	+	−	−	−	−	−	−
4	6 immune	−	−	−	−	−	−	−	−

Intradermal inoculations were used in the experiments.
+ indicates a visible reaction at the site of inoculation.
− indicates the absence of a visible reaction at the site of inoculation.

Heat.—Virus III is very sensitive to heat. The results of two experiments which were performed to determine the effect of heat upon the virus are summarized in Table II. Portions of fresh testicular emulsions containing active virus which produced visible skin lesions in dilutions of 1:1,000 were heated various lengths of time at different temperatures. Then a small quantity of each portion was injected into the skin of the same normal rabbit and also into the skin of the same immune rabbit. No visible reactions were obtained in the immune rabbit. Definite reactions occurred, however, in the skin of the normal rabbit at the sites of inoculation of the unheated virus and the virus heated at 45°C. for 30 minutes. The portions of virus heated 10, 20, 30, 40, and 50 minutes at 55°C. and 30 minutes at 65°C. respectively produced no visible reaction in the skin of the normal rabbit.

TABLE III.

Summary of the Results of the Experiments Showing the Effect of Physiological Salt Solution, Locke's Solution, and Glycerol upon the Viability and Activity of Virus III.

Rabbit	Unfiltered testicular virus, 1, preserved in physiological salt solution unsealed on ice.		Unfiltered testicular virus, 2, preserved in Locke solution on ice. Sealed with vaseline.	
	2 days	5 days	23 days	
Immune	−	−	−	
Normal	+	−	−	
	Unfiltered testicular virus, 3, preserved in Locke solution unsealed on ice.		Unfiltered testicular virus, 3, preserved in Locke solution on ice. Sealed with vaseline.	
	30 days		30 days	
Immune	−		−	
Normal	−		−	
	Unfiltered testicular virus, 4, preserved in Locke solution on ice. Sealed with vaseline.		Unfiltered testicular virus, 4, preserved in Locke solution and 40 per cent glycerol on ice. Sealed with vaseline.	
	54 days		54 days	
Immune	−		−	
Normal	−		−	
	Berkefeld filtrate of testicular virus, 5, preserved in Locke solution unsealed on ice.		Berkefeld filtrate of testicular virus, 5, preserved in Locke solution on ice. Sealed with vaseline.	
	28 days	61 days	28 days	61 days
Immune	−	−	−	−
Normal	−	−	+	−
	Berkefeld filtrate of testicular virus, 6, preserved in Locke solution unsealed on ice.		Berkefeld filtrate of testicular virus, 6, preserved in Locke solution on ice. Sealed with vaseline.	
	17 days	52 days	17 days	52 days
Immune	−	−	−	−
Normal	−	−	+	−
	Berkefeld filtrate of testicular virus, 7, preserved in Locke solution on ice. Sealed with vaseline.		Berkefeld filtrate of testicular virus, 7, preserved in Locke solution and 40 per cent glycerol on ice. Sealed with vaseline.	
	24 days		24 days	
Immune	−		−	
Normal	−		+	

Intradermal inoculations were used in these experiments.
+ indicates a visible reaction at the site of inoculation.
− indicates absence of a visible reaction at the site of inoculation.

Physiological Salt Solution, Locke's Solution, and Glycerol.—It seemed of interest to ascertain under what conditions the virus can be best preserved in an active state *in vitro*. Therefore the effects of physiological salt solution, Locke's solution, and glycerol upon the viability and activity of the virus were observed. These observations are summarized in Table III. Under certain conditions the virus evidently deteriorates rapidly when removed from the body and quickly ceases to produce visible lesions in the skin. Even though the virus deteriorates rapidly *in vitro*, some of it remains viable in physiological salt solution and in 40 to 50 per cent glycerol various lengths of time (Text-fig. 1) and is recoverable in a highly active state after several passages through rabbits.

The virus (Table III) in the unfiltered testicular emulsions, with or without the addition of glycerol, sealed or unsealed, after a short time ceased to produce visible skin reactions. The virus in the Berkefeld filtrates of the testicular emulsions sealed with vaseline, however, continued to produce definite skin reactions longer than did the virus in the unfiltered emulsions preserved under similar conditions. The difference in the preservation of the virus under these conditions becomes more evident when the difference in its concentration in the filtered and unfiltered fresh emulsions is considered. The fresh unfiltered material was active in most instances in dilutions of 1:1,000 while the filtrates were active only in the undiluted portion or in dilutions of 1:10. The addition of glycerol to the filtrates before they were sealed with vaseline also seemed to aid in the preservation of the virus. The data obtained so far indicate that the best method of preserving the virus in an active state is to filter the testicular emulsions, add glycerol to the filtrates up to 40 per cent of the total volume, seal with vaseline, and store on ice.

Study of the Immunity Produced by the Virus.

Active Immunity.[3]

One of the striking characteristics of most of the so called filterable viruses is that an infection caused by them leads to the appear-

[3] In the experiments reported under Active Immunity, a viable virus was always used. This was carefully controlled even when no note of it appears in the report of the individual experiments.

TABLE IV.

Summary of the Results of Studies upon the Active Immunity Produced by Virus III.

Rabbit	Material used for immunizing dose	Site of inoculation of immunizing dose	Reaction to immunizing dose	Time between immunizing & testing doses	Material used for testing dose	Site of inoculation of testing dose	Result
1	Fresh testicular virus	Testicles & skin	Visible reaction testicles & skin	4 days	Fresh testicular virus	Skin intradermally	Partially immune
2	"	"	"	5 "	"	"	Immune
3	"	Skin intradermally	Visible reaction	10 "	"	"	"
4	"	"	"	14 "	"	"	"
5	"	"	"	60 "	"	"	"
6	Testicular virus preserved on ice 42 days in 40 per cent glycerol	"	No visible reaction	18 "	"	"	"
7		"	"	18 "	"	"	"
8		"	"	18 "	"	"	"
9	Normal testicle preserved in 40 per cent glycerol on ice 31 days	"	"	18 "	"	"	Not immune
10		"	"	18 "	"	"	"
11		"	"	18 "	"	"	"
12		"	"	18 "	"	"	"
13		"	"	18 "	"	"	"
14	Fresh testicular virus	Brain	Fever	25 "	"	"	Immune
15	"	"	"	29 "	"	"	"
16	"	"	"	29 "	"	"	"
17	Blood containing active virus	Intravenously	No visible reaction	16 "	"	"	"
18	"	"	"	16 "	"	"	"
19	"	"	"	14 "	"	"	"
20	"	"	"	14 "	"	"	"
21	"	Testicles	"	16 "	"	"	"
22	"	"	"	16 "	"	"	"
23	"	"	"	14 "	"	"	"
24	"	"	"	14 "	"	"	"
25	Fresh testicular virus	Intravenously	"	17 "	"	"	"
26	"	Nose	"	20 "	"	"	"
27	"	"	"	20 "	"	"	"
28	"	"	"	28 "	"	"	"
29	"	"	"	28 "	"	"	"
30	"	"	"	28 "	"	"	"
31	"	Smeared on unshaved skin of ears	"	23 "	"	"	Not immune
32	"		"	23 "	"	"	"
33	"		"	23 "	"	"	"
34	"	Testicles & skin	Visible reaction	6 months	"	"	Immune

All testicular virus used for immunizing and testing doses was controlled with animals known to be normal and immune.

The virus was demonstrated in the blood said to contain active virus by passage through rabbits' testicles.

The testicular virus preserved in glycerol was still viable as an active virus was recovered by passage through rabbits' testicles.

ance in recovered animals of a marked lasting immunity, or refractory state. Therefore it seemed of interest to determine if the animals which had recovered from a primary inoculation of the virus under investigation were refractory to subsequent inoculations of the same virus. It was soon found that testicular emulsions containing active virus when injected into rabbits regularly produced an immunity which did not follow inoculations with emulsions of normal testicles.

Time of Appearance of the Immune or Refractory State.—The time of appearance of the refractory state in animals probably depends on a number of factors; for example, the amount, condition, and mode of administration of the virus. It seemed of interest to determine how soon a refractory state can be established in rabbits following injections of large amounts of Virus III. 5 days were found to be the shortest length of time within which a rabbit becomes immune to the virus.

Rabbit 1 (Table IV) received in each testicle 1 cc. of a fresh testicular emulsion containing Virus III and also 0.2 cc. of the same emulsion intradermally. Typical reactions occurred in the testicles and skin. 4 days after the primary inoculations the rabbit was reinoculated intradermally with active Virus III. A mild accelerated reaction appeared at the site of reinoculation. Rabbit 2 was treated in a similar manner with the exception that the reinoculation of the skin was made 5 days after the primary injection. While definite reactions occurred after the primary injections, none was observed following the second intradermal inoculation.[4]

Immunity Following Intradermal Inoculations of Active Virus III.—The skin of rabbits became immune within a short time after intradermal injections of small amounts of active virus.

Rabbits 3, 4, and 5 (Table IV) received intradermally 0.2 cc. of a fresh testicular emulsion containing active Virus III. Typical reactions followed the primary inoculations. The skin of these animals, however, was immune to active virus after 10, 14, and 60 days, respectively.

Immunity Following Intradermal Inoculations of Virus III Preserved in Glycerol.—A glycerolated virus, which was still viable, im-

[4] The virus used in each of the immunity experiments was controlled on normal and on immune rabbits.

munized rabbits when injected intradermally. The results of this experiment are summarized in Table IV.

Immunity Following Intracerebral Inoculations of Virus III.— Small amounts of fresh testicular emulsions containing active virus when injected into the brains of young rabbits (1,000 gm.) caused febrile reactions more severe than those produced in control animals by intracerebral inoculations of emulsions of normal testicles. The rabbits did not die after the intracerebral inoculations and the animals which received the virus developed an immunity.

Rabbits 14, 15, and 16 (Table IV) were inoculated intracerebrally with 0.2 cc. of a fresh testicular emulsion containing active Virus III. Sharp febrile reactions occurred 3 to 5 days after the injections. The animals were reinoculated intradermally with active Virus III 25 to 29 days later. No reactions followed the second inoculations.

Immunity Following Intravenous Inoculations of Active Virus III.— A refractory state was established in animals following intravenous injections of the virus.

Rabbits 17, 18, 19, 20, and 25 (Table IV) were inoculated intravenously either with fresh infective rabbit blood or with fresh testicular emulsion containing active virus. 14 to 17 days later the animals were reinoculated intradermally with fresh active Virus III without the development of any visible reactions.

Immunity Following Intranasal Instillations of Active Virus III.— The instillation of active Virus III into the nostrils of rabbits was followed by the appearance of an immunity in the skin of the animals against the virus. No lesions were observed around the external nasal orifices following the intranasal inoculations of the virus. Lesions might have occurred in the mucous membranes inaccessible for examination in living animals. This could not be determined, however, as it was impossible to sacrifice a rabbit for an examination of the nasal mucous membranes and at the same time save the animal for future immunity work. That the virus instilled into the nose had produced a reaction somewhere in the animals was evidenced by the immunity that subsequently developed.

0.5 cc. of a fresh testicular emulsion containing active Virus III was instilled into each nostril of Rabbits 26, 27, 28, 29, and 30 (Table IV). The nasal mucous membranes were not injured at the time of the inoculations and no reactions were noticed in the animals during the 2 weeks of observation. The skin of the rabbits was refractory to active virus 20 to 28 days later. The experiment was controlled by rubbing active virus on the unshaved skin of the ears of Rabbits 31, 32, and 33 (Table IV). The skin was not shaved because shaving alone often injures the skin enough to permit the entrance of the virus into the body. The skin of the control animals, which received applications of the virus on the unshaved skin of the ears, was not refractory to the virus 23 days later.

Duration of the Immunity.—At present it is impossible to state how long the immunity persists in animals. It is known, however, that the immunity lasts for at least 6 months, as all the rabbits, when tested 6 months after the primary inoculations, were still immune to the virus.

Immunological Relationship of Three Strains of the Virus under Investigation.—Three strains of the virus under investigation have been shown experimentally to be immunologically identical. These strains are designated Virus III, Virus IV, and Virus V.

Three rabbits each were inoculated intradermally with Virus III, Virus IV, and Virus V. A typical reaction followed the primary inoculations in all the animals. 2 weeks later these rabbits were reinoculated intradermally with active Virus III, the most vigorous strain, and in no instance was there a reaction at the site of the second inoculation.

Immunological Relationship between Vaccine Virus and Virus III.—Experiments have failed to demonstrate an immunological relationship between vaccine virus and Virus III.

Eight rabbits which had been previously shown by test to be immune to Virus III, were inoculated on the scarified skin with vaccine virus. An eruption typical of vaccinia occurred in each rabbit. Five rabbits which had been previously shown by test to be immune to vaccine virus were inoculated intradermally with active Virus III. A typical reaction occurred in each rabbit at the site of the inoculation. The vaccine virus and Virus III used in this experiment were controlled in normal and immune rabbits.

Relationship between Virus III and the Virus of Symptomatic Herpes.[5]—Virus III seems to be more closely allied to the virus of symptomatic herpes than to vaccine virus. The intranuclear changes seen

[5] Two strains of the virus of symptomatic herpes were supplied by Dr. Flexner.

in cells injured by Virus III and the virus of herpes are almost identical. In spite of this similarity, definite differences in the behavior of the two viruses have been observed. Virus III never kills young rabbits when injected intracerebrally, does not produce vesicles following skin inoculations, gives more constant results when injected intradermally than does the herpes virus, and produces only a mild reaction when inoculated upon a scarified cornea.

Furthermore, the two viruses are immunologically distinct, as shown by the following experiments.

Rabbit A, immune to Virus III for 30 days, received an intracerebral inoculation of 0.25 cc. of a strong herpes virus. The rabbit showed the usual symptoms of encephalitis and died on the 3rd day after the inoculation.

Rabbit B, immune to Virus III for 70 days, received an intracerebral inoculation of 0.25 cc. of a weak herpes virus. The animal showed the usual symptoms of encephalitis, had a temperature of 108°F. on the 7th day, and died on the 8th day following the inoculation.

Rabbit C (1,000 gm.) received intracerebrally 0.25 cc. of a weak herpes virus which had been preserved in glycerol on ice for 3 months. On the 4th day after inoculation the animal's temperature rose to 104°F., remained high for 4 days, during which time the rabbit was sick and ataxic. The animal recovered and 10 days later proved equally susceptible as control animals to Virus III injected intradermally.

Rabbit D (1,800 gm.) showed the usual reaction following the intradermal inoculation of Virus III. 14 days later the animal was inoculated intradermally with active Virus III on one side of the body and with active herpes virus on the other side. No reaction was observed at the site of the reinoculation of Virus III. Typical lesions, however, occurred at the site of the inoculation of the herpes virus.

Absence of Immunity in Rabbits Following a Single Intradermal Injection of Virus III Which Had Been Heated 10 Minutes at 55° C.—A single intradermal injection of Virus III, which had been heated 10 minutes at 55° C., was not followed by any appreciable immunity in rabbits, while one of the unheated virus was regularly followed by a refractory state.

Passive Immunity.

Failure of Intravenous Injections of Immune Rabbit Serum to Protect Normal Rabbits against Virus III.—After it was established that rabbits can be immunized actively by injections of viable virus, it was considered desirable to determine if animals can also be protected

passively against the virus by injections of serum from immune animals. The serum was collected from immune rabbits, 2 or 3 weeks after the immunizing dose of virus, and contained virucidal properties as will be shown further on. A number of rabbits received intravenous injections of 5 to 10 cc. of the immune serum and 24 hours later were inoculated intradermally with active Virus III. The rabbits which had received the serum, and normal control animals, proved equally susceptible to the virus, while actively immunized rabbits were refractory. It has not been determined whether the negative outcome of these experiments was due to the injection of too small an amount of serum, to the length of time allowed between the injection of the serum and the inoculation of the virus, or to an impossibility of demonstrating passive immunity in rabbits to Virus III following the intravenous injection of immune serum. Even though a definite explanation of the negative results is lacking, it is, nevertheless, very evident that rabbits were not protected by the intravenous injection of immune serum under the conditions outlined in the experiments.

Virucidal Action of Immune Rabbit Serum.

Neutralization of Virus III by Immune Rabbit Serum in Vitro.— The immune rabbit serum, which did not protect normal rabbits when 5 to 10 cc. of it were given intravenously, was found to be virucidal when mixed with the virus *in vitro*.

Various mixtures of the virus and sera with controls were set up and incubated at 37° C. for 2 hours as indicated in Table V. Then a small quantity of each mixture was injected intradermally into the same normal rabbit. The rabbit was examined daily for 2 weeks for the appearance of reactions at the sites of the injections.

The tests showed that immune rabbit sera neutralized the virus, while normal rabbit sera, Locke's solution, and physiological salt solution did not. The results were striking, as witness the skin of the rabbit (Fig. 3) and the summary of three experiments in Table V. The results were the same when complement was included or excluded from the mixtures *in vitro*. At present it is not known whether complement is essential for the neutralization of the virus, as the

results of the various mixtures after incubation must be determined in the skin of living animals where complement may be supplied

TABLE V.

Summary of the Results of Three Experiments Showing that Immune Rabbit Serum Neutralizes Virus III in Vitro.

Rabbit	Mixtures incubated 2 hrs. at 37°C. and then small quantities of each were injected intradermally into rabbits	Dilutions of the virus									
		1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512	1-1024
3	.5cc. (salt solution and complement) + .5cc. various dilutions of virus	+	+	+	+	+					
	.5cc. (normal rabbit serum, 1, and complement) + .5cc. various dilutions of virus	+	+	+	+	+					
	.5cc. (immune rabbit serum, 1, and complement) + .5cc. various dilutions of virus	−	−	−	−	−					
4	.5cc (salt solution and complement) + .5cc. various dilutions of virus			+	+	+	+	+	+	+	+
	.5cc. (normal rabbit serum, 2, and complement) + .5cc. various dilutions of virus			+	+	+	+	+	+	+	+
	.5cc. (immune rabbit serum, 5, and complement) + .5cc. various dilutions of virus			−	−	−	−	−	−	−	−
6	.5cc. fresh immune rabbit serum, 7, + .5cc. various dilutions of virus	−			−			−			−
	.5cc. inactivated immune rabbit serum, 7, + .5cc various dilutions of virus	−			−			−			−
	.5cc. Locke solution + .5cc. various dilutions of virus	+			+			+			+

Normal rabbit serum, 1, and immune rabbit serum, 1, came from the same animal, the former before immunization, the latter 14 days after an intradermal injection of the virus.
+ indicates a visible lesion at the site of inoculation.
− indicates absence of a visible lesion at the site of inoculation.

for the completion of the reaction if this has not already been accomplished *in vitro*.

Neutralization of Virus III Locally in Vivo by Immune Rabbit Serum.—Doubt arose as to whether the neutralization of the virus

by immune serum was completed *in vitro* or whether a part or all of the reaction occurred locally in the skin of the normal rabbit following the injections of the various mixtures described in the preceding experiments. It was early found that mixtures of virus and sera which were injected immediately into the skin of rabbits gave results identical with those obtained following injections of similar mixtures which had been incubated 2 hours at 37° C. (Tables

TABLE VI.

Summary of the Results of One Experiment Showing that Immune Rabbit Serum Neutralizes Virus III in Vivo When the Serum and the Virus Are Injected into the Same Part of the Skin at or about the Same Time.

RABBIT A

Right side		Left side	
Front	Result	Front	Result
Small amount of fresh virus injected intradermally.	+	.5cc. fresh virus + .5cc. Locke solution mixed and a small amount of the mixture injected immediately intradermally.	+
Rear		Rear	
An area of skin 3 cm. in diameter was infiltrated with 1.5cc. of inactivated immune rabbit serum. 10 min. later a small amount of fresh virus was injected intradermally in the center of the infiltrated area.	—	.5cc. fresh virus + .5cc. inactivated immune rabbit serum mixed and a small amount of the mixture injected immediately intradermally.	—

+ indicates the usual visible reaction following an intradermal injection of fresh active virus.

— indicates the absence of a visible reaction at the site of injection after 48 hours.

V and VI). Furthermore, it was found that, if a small area in the skin of a normal rabbit was infiltrated with immune serum and then 10 minutes later active virus was injected in the center of the infiltrated area, no lesion occurred. Although no lesion occurred in the area of skin protected by the immune serum, a typical reaction appeared at the site of the injection of virus into a control area of the skin only a short distance away (Table IV).

Identification of Virus III.

Virus III was recovered by injecting blood of varicella patients into the testicles of rabbits and then making repeated transfers at 4 day intervals from rabbit to rabbit by means of testicular inoculations. The method employed in recovering the virus, the fact that more than half of the experiments which were performed in attempting to recover the virus resulted negatively, the macroscopic and microscopic lesions produced in rabbits by the virus, led us to infer that we were not improbably working with the etiological agent of chicken-pox. It was realized that the final proof that the virus is the etiological agent of varicella was lacking and in a previous paper[1] we expressly stated that: "Further evidence must be obtained, however, before one can think and speak definitely of this virus as the etiological agent of varicella." A better understanding of the behavior of the virus in animals and better methods of working with the virus were necessary before we could rely on the results of experiments performed to identify the virus. A description of the necessary methods and the behavior of the virus in normal and immune animals have been described above. With this information we were then prepared to undertake the identification of Virus III. The results of this work follow.

Failure of Intravenous Injections of Serum and Whole Blood from Convalescent Chicken-Pox Patients to Protect Normal Rabbits against Virus III.—Neither 5 to 10 cc. of whole blood nor 5 to 10 cc. of serum from patients convalescent from chicken-pox, when injected intravenously, protected normal rabbits against Virus III inoculated intradermally 24 hours after the administration of the serum or blood. This was considered to be in accord with experiments reported above in which it was shown that intravenous injection of immune rabbit serum which neutralized the virus *in vitro* would not protect normal animals against Virus III inoculated intradermally.

Failure of Serum from Patients Convalescent from Chicken-Pox to Neutralize Virus III in Vitro.—Since immune rabbit serum neutralizes Virus III *in vitro* and since a reliable technique has been devised by which this neutralization can be demonstrated, it seemed wise to determine if the serum of patients convalescent from vari-

cella possesses any demonstrable virucidal properties for Virus III or if the serum collected during convalescence neutralizes more virus than does the serum collected during the first 2 days of the disease. The results of these experiments showed that sera from two normal adults and from fourteen patients convalescent from varicella have no demonstrable neutralizing effect upon Virus III *in vitro*. Furthermore, no difference could be detected in the sera collected from four patients during the disease and during convalescence. We felt that the serum from a patient convalescent from varicella should neutralize Virus III *in vitro*. When it was found, however, that the serum did not neutralize the virus, it occurred to us that the virus might be too concentrated or too active to be affected by a small amount of immune bodies that might appear in the serum of convalescents.

Failure of Rabbits to Be Actively Immunized against Virus III by Injections of Whole Blood, Vesicle Fluid, or Nasal Washings from Varicella Patients.—Since it was known that the virus is present in small amounts of blood taken from a rabbit 4 days after intratesticular inoculation with Virus III, since an active immunity promptly appears in rabbits inoculated with small amounts of this blood, and since there is evidence that the blood of varicella patients contains the etiological agent of chicken-pox, we decided to see if animals could be actively immunized against Virus III by injections of fresh blood from patients with chicken-pox early in the disease. In this manner we hoped to establish some relationship between Virus III and the etiological agent of varicella. Vesicle fluid and nasal washings were also injected into a few animals. Control animals received similar amounts of fresh blood from normal humans. After the inoculations the animals were kept 21 to 67 days and then the presence of an immunity in them was determined by means of intradermal inoculations of various dilutions of active Virus III. In each experiment the virus was controlled in normal and immune animals. Eleven experiments were performed in which 39 test and 23 control animals were employed. The results of these experiments are summarized in Table VII. The percentage of immune rabbits was the same in the control as in the experimental animals, 26 per cent. Experiments 3, 10, and 11 taken alone might be in-

TABLE VII.

Summary of Eleven Experiments Showing that Rabbits Could Not Be Actively Immunized against Virus III by Injections of Blood, Nasal Washings, or Vesicle Fluid from Varicella Patients. These Experiments Also Show that a Certain Number of Rabbits Are Refractory to Virus III Regardless of the Materials Previously Inoculated.

		Test animals						Control animals						
Experiment	Patient	Number rabbits	Material injected	Site of injection	Time before testing immunity	Number immune	Number not immune	Normal man	Number rabbits	Material injected	Site of injection	Time before testing immunity	Number immune	Number not immune
1	Rev.	3	Blood	Testicles	21 days	2	1	F	3	Blood	Testicles	21 days	1	2
2	Rec.	3	"	"	21 "		3	T.	2	"	"	21 "		2
		2	Nasal washings	Skin	21 "		2							
		1	Vesicle fluid	"	21 "	1								
3	Bo.	3	"	"	25 "		3	R.	2	"	"	25 "		2
		1	Nasal washings	"	25 "		1							
		3	Blood	Testicles	25 "	3								
4	Fer.	5	"	"	26 "		5	T	5	"	"	26 "		5
		2	Vesicle fluid	Skin	32 "		2							
5	Ric.	3	Blood	Testicles	41 "		3	T.	3	"	"	41 "	1	2
6	Ka.	1	"	Vein	30 "	1		T.	1	"	Vein	30 "		1
7	Da.	1	Vesicle fluid	Skin	48 "		1							
8	Co.	2	"	"	45 "		2							
9	Pe.	2	"	"	41 "		2	T.	2	"	"	54 "	2	
		2	Blood	Testicles	54 "		2		2	"	Testicles	54 "	2	
		2	"	Vein	54 "	1	1							
10	He.	1	"	Testicles and vein	67 "	1		T.	1	"	Testicles and vein	67 "		1
11	A & B	2	"	"	67 "	1	1	F.	2	"	"	67 "		2
Total						10	29	Total					6	17
Per cent immune							25.6	Per cent immune						26.08

Immunity determined by intradermal inoculations of 0.2 cc. various dilutions of fresh active Virus III.
The percentage of animals immune was the same in the test and the control animals.
In Experiment 3 the test animals which received blood were caged together twenty-five days.
In Experiment 9 the control animals were caged together fifty-four days.

terpreted as evidence in favor of Virus III being the etiological agent of chicken-pox, but when all eleven of the experiments are considered together, no evidence can be obtained from them in support of such a deduction.

Further study of the experiments reveals that the failure of 26 per cent of the rabbits to react to Virus III was not dependent upon

TABLE VIII.

Summary of an Experiment Showing that the Serum of One of Six Stock Rabbits Neutralized Virus III in Vitro, and that the Virucidal Properties of the Sera Usually Parallel the Intradermal Tests with the Virus.

Rabbit	Neutralization of Virus III by the serum of the rabbit before inoculation with blood	Inoculation of blood into testicles and vein of rabbit	Time allowed between inoculation of blood and second neutralization tests	Neutralization of Virus III by serum of the rabbit after inoculation with blood	Immunity of rabbit as determined by intradermal inoculation of Virus III
A	Yes	From patient with varicella	67 days	Yes	Yes
B	No	"	"	"	"
C	"	"	"	No	No
D	"	From normal adult	"	"	"
E	"	"	"	"	"
F	"	"	"	"	"

The serum from one of six rabbits, A, taken from stock neutralized Virus III.
Sixty-seven days later two sera, A and B, neutralized Virus III.
The results of the second neutralization experiment were identical with those obtained by intradermal injection of Virus III.

the previous inoculations of blood, vesicle fluid, or nasal washings. The control animals in Experiments 2, 4, 5, 6, 9, and 10 received blood from the same normal man. In spite of this, the four control animals in Experiment 9 were immune and only one of the twelve rabbits in Experiments 2, 4, 5, 6, and 10 which received blood from the same subject was found to be immune. It is interesting to note that the four control rabbits in Experiment 9 were caged together for 54 days and that the three test animals which received blood in Experiment 3 and which were later found to be immune were also caged together for 25 days. In our previous work of making routine transfers we had found that only about 15 per cent of 200 young stock

rabbits (1,800 gm.) failed to react to intradermal inoculations of Virus III and were surprised to find so many refractory rabbits in these experiments.

As the work progressed and many refractory animals were found among the controls, it became necessary to ascertain in some way if normal stock rabbits were refractory before the experiments were begun. The only way to accomplish this was to determine the virucidal properties of each rabbit's serum for Virus III *in vitro* before inoculating the animal with blood, determine it again before testing the skin of the animal with Virus III, and then compare the results obtained by the two methods. The results of one such experiment are summarized in Table VIII. The animals in this experiment are identical with those in Experiments 10 and 11 in Table VII. From these results it can be seen that the sera of certain stock rabbits neutralize Virus III *in vitro*, that the presence of an immunity in a rabbit as evidenced by the failure of a visible reaction to follow the intradermal inoculation of Virus III closely parallels that evidenced by the virucidal properties of the rabbit's serum for Virus III *in vitro*, and that the immunity shown to exist in the test and control animals in Table VII was not dependent upon the blood, nasal washings, or vesicle fluid injected into the rabbits.

The power of a rabbit's serum to neutralize Virus III *in vitro* does not necessarily parallel the failure of a reaction to follow an intradermal inoculation of Virus III in the same animal. The virucidal properties of a rabbit's serum may disappear in 3 to 6 months. The animal remains refractory, however, to the virus. This phenomenon is known to exist in rabbits immune to vaccine virus. If young rabbits are chosen, few refractory ones are encountered and the skin reactions and the power of the serum to neutralize the virus *in vitro* usually parallel each other.

Twenty sera from stock rabbits of different ages have been examined for virucidal properties for Virus III. Four of these sera, 20 per cent, neutralized the virus *in vitro*. The animals whose sera neutralized the virus *in vitro* were subsequently found to be refractory when the skin was tested with active Virus III.

Inoculation of Active Virus III into Animals Other than Rabbits.—Guinea Pigs: Two guinea pigs received intradermal inoculations of

active Virus III. No visible reaction appeared in either pig during 3 weeks of observation.

White Mice: Six white mice received intraperitoneal injections of 0.25 to 1.25 cc. of active Virus III. During 10 days of observation all the mice remained well and active.

Monkeys: Each of two monkeys (*Macacus rhesus*) received an intravenous injection of 2 cc. of active Virus III in 8 cc. of physiological salt solution, and an intradermal inoculation of 0.2 cc. of the same virus. No visible skin reactions, no significant changes in the temperature or in the blood count occurred in either monkey during 3 weeks of observation.

Humans: Two men, physicians, volunteered for intradermal inoculations of active Virus III. One of the men had had varicella in childhood, the other had never suffered from varicella. Each volunteer received 0.2 cc. of active virus intradermally in two places on the left upper arm. The volunteer who had not suffered from varicella experienced no general reaction and only a mild local one consisting of redness and tenderness in the immediate vicinity of the inoculation which disappeared entirely in 3 days. The man who had had varicella in childhood experienced a more severe reaction. 8 hours after the inoculation a chilly sensation, headache, backache, and general malaise were noticed. The arm at the site of the inoculation became red, swollen, tender, and painful. The general reaction disappeared after 48 hours. The local reaction increased in intensity for 36 hours; the redness, swelling, and tenderness extended half way down the forearm. The axillary glands were swollen and tender. The local reaction gradually subsided and disappeared in 5 days. In neither of the subjects did a vesicle or an open lesion appear at the site of the inoculation. No generalized eruption appeared in either man. Both individuals were still well a month after the injections. The blood serum of neither man neutralized Virus III *in vitro* before the inoculations and is now being studied at weekly intervals for the occurrence of virucidal properties.

DISCUSSION.

From the work reported previously[1] and that presented in this paper it is evident that an active, transmissible agent is being studied which partakes of the characters of the so called filterable viruses. By immunological tests in humans and rabbits it has been impossible to obtain any evidence that this virus is associated in patients with the manifestations of varicella. On the other hand, the studies do bring evidence that we are dealing with a specific previously unknown virus which is quite distinct from vaccine virus and the virus of symptomatic herpes. There is no evidence that Virus III is the etiological agent of rabbit snuffles. The virus of infectious myxomatosis is the only filterable virus indigenous to rabbits concerning which we have been able to find reports in the literature. This virus was first reported by Sanarelli[6] and further described by Splendore[7] and Moses.[8] It produces fatal myxomatous tumors in rabbits, is filterable, and can be transmitted indefinitely from rabbit to rabbit. The exact source and nature of Virus III and its relationship to human or rabbit diseases remain to be determined.

CONCLUSIONS.

1. The intradermal method of inoculating Virus III, a hitherto unknown filterable virus producing lesions in rabbits, gives more reliable results than those obtained by smearing the virus on the scarified skin.

2. Virus III, heated 10 minutes at 55°C., will not produce visible reactions in the skin of rabbits.

3. Virus III passes through Berkefeld N and V filters.

4. The data obtained so far indicate that the best method of preserving Virus III in an active state is to filter the testicular emulsions containing the virus, add glycerol to the filtrate up to 40 per cent of the total volume, seal with vaseline, and store on ice.

5. Viable Virus III produces a definite immunity in rabbits which persists for at least 6 months. The immunity follows intradermal,

[6] Sanarelli, G., *Centr. Bakt., 1. Abt.*, 1898, xxiii, 865.
[7] Splendore, A., *Centr. Bakt., 1. Abt., Orig.*, 1909, xlviii, 300.
[8] Moses, A., *Mem. Inst. Oswaldo Cruz*, 1911, iii, 46.

intratesticular, intravenous, intracerebral, or intranasal inoculations of the virus.

6. A single intradermal injection of Virus III, which has been killed by heat, will not produce a demonstrable immunity in rabbits.

7. No passive immunity to Virus III could be demonstrated in rabbits which had received intravenous injections of 5 to 10 cc. of immune rabbit serum 24 hours previously.

8. Immune rabbit serum neutralizes Virus III either *in vitro*, or locally in a rabbit's skin when the immune serum and the virus are injected into the same part of the skin at or about the same time.

9. Three strains of the virus under investigation are immunologically identical.

10. Virus III and vaccine virus are immunologically distinct.

11. Virus III and the virus of symptomatic herpes are immunologically distinct.

12. No passive immunity to Virus III could be demonstrated in rabbits which had received intravenous injections of 5 to 10 cc. of serum or whole blood from patients convalescent from varicella.

13. Sera from two normal adults and from fourteen patients convalescent from varicella did not neutralize Virus III *in vitro*.

14. Rabbits could not be actively immunized against Virus III by injections of whole blood, vesicle fluid, or nasal washings from patients with varicella.

15. Four of twenty sera collected from stock rabbits of different ages, 20 per cent, neutralized Virus III *in vitro*. The animals whose sera neutralized Virus III failed to show a reaction at the site of intradermal inoculations with the same virus. About 15 per cent of 200 young stock rabbits (1,800 gm.) used in routine transfers were found to be refractory to Virus III, as evidenced by a failure to react to intradermal inoculations of the virus.

16. No susceptibility to Virus III was observed in guinea pigs, mice, or monkeys.

17. A volunteer who had never suffered from varicella experienced no general reaction and only a mild local one following an intradermal inoculation of Virus III. A volunteer who had had chicken-pox in childhood experienced a moderate general action, *viz.*, fever, headache,

backache, and general malaise, and also a moderate local reaction, *viz.*, redness, swelling, tenderness, and pain, following an intradermal inoculation of Virus III.

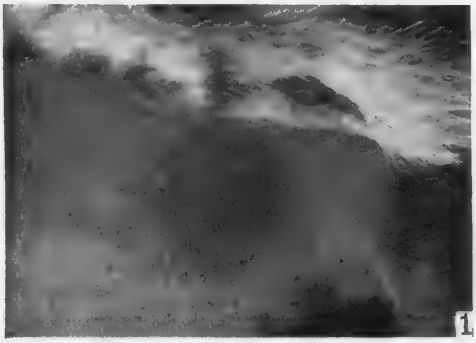
18. The study of the immunological reactions has failed to bring any evidence that the virus under investigation bears an etiologic relationship to varicella.

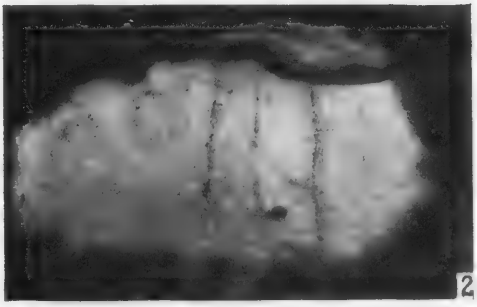
EXPLANATION OF PLATE 38.

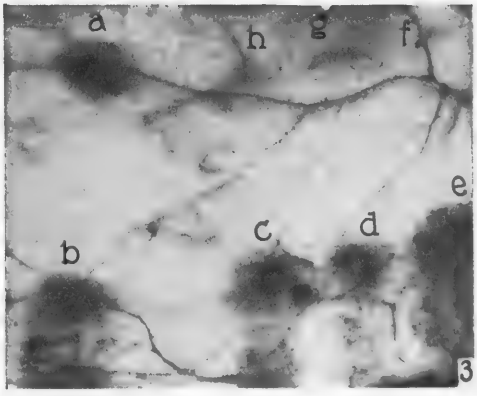
FIG. 1. Usual reaction in the skin of a rabbit following the intradermal inoculation of active Virus III. Photograph made 5 days after the inoculation.

FIG. 2. Usual reaction in the skin of a rabbit when active Virus III is smeared along the lines of scarification. Five lines were made on the skin. Lines 2 and 4 were inoculated. Lines 1, 3, and 5 were not inoculated. Photograph made 5 days after inoculation.

FIG. 3. Photograph of rabbit's skin in which the mixtures of Experiment 1 in Table V were tested. *a* and *b* are sites of injection of mixtures of salt solution and dilutions of the virus; *c*, *d*, and *e* are sites of injection of mixtures of normal rabbit serum and dilutions of the virus; *f*, *g*, and *h* are sites of injection of mixtures of immune rabbit serum and dilutions of the virus. The immune serum neutralized the virus as shown by the failure of lesions to appear at *f*, *g*, and *h*.







(Rivers and Tillett: Varicella.)

UNIV OF MICH

THE TRANSMISSION AND TREATMENT OF INFECTIOUS OPHTHALMIA OF CATTLE.

By F. S. JONES, V.M.D., and RALPH B. LITTLE, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

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In a previous publication[1] an acute inflammation of the eye of dairy cows was described. From a series of cases a characteristic paired bacillus was isolated. It was possible to reproduce the disease with pure cultures of this organism. It was clearly established that in certain cases the bacilli persisted in the ocular secretions after the acute symptoms had subsided.

History of the Infection.

Cows from a midwestern state suffering with acute ophthalmia had been introduced into the herd early in September, 1922. A considerable number of cases occurred in cows in two adjoining barns during September and October. With the onset of cold weather the epidemic subsided but sporadic cases occurred during the colder months. The disease again became epidemic during the months of July, August, September, October, and November, 1923. New cases occurred as late as December. On the whole the inflammations were less severe during the second outbreak, but cases of considerable severity were not uncommon. In our previous paper we called attention to the possibility of a recurrence of the disease in epidemic form since five of ten cows reexamined 3 or 4 months after our first observation still carried the organism. At that time attention was also called to the possibility of transmission by flies. Certain facts seemed to incriminate the house-fly with the spread of infection. Flies feed readily on the exudate at the inner canthus of the affected

[1] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1923, xxxviii, 139.

eyes. While direct infections doubtless occur, in the main new cases appear irregularly. The fact that the epidemics reached their maximum during the warmer months also suggests fly transmission. The transmission of the Koch-Weeks bacillus of human ophthalmia in Egypt by flies has been reported by Budd, Laveran, Howe, Biskra, Koch, and others.[2,3] Howe called attention to the prevalence of the disease during the season when flies were most numerous. He further asserted that the same organism which occurred in the conjunctival exudate could be found on the feet of flies. We attempted by a series of experiments to test the hypothesis that flies transmit the disease.

EXPERIMENTAL.

At first we attempted to experiment with flies caught feeding upon the exudate about the eyes of spontaneous cases. Too few were obtained in a viable condition to afford sufficient material. Flies were therefore fed artificially on exudate containing large numbers of viable diplobacilli.

Experiment 1.—Six house-flies were caught feeding on the eyes of spontaneous cases. Within ½ hour they were placed in a hemispherical cage and applied over the eye of a cow. The flies refused to feed on the lacrimal fluid but some came in contact with the eyeball. The eye remained normal and diplobacilli were not found in the lacrimal fluid.

In the next series of experiments flies were captured in rooms in the laboratory. They were given only water for 10 or 12 hours before feeding. Exudate from a number of spontaneous cases was obtained on sterile cotton swabs. The material was prevented from drying by the addition of a little sterile bouillon. Flies were permitted to feed on this material shortly after it had been obtained.

Experiment 2.—Sept. 20, 1923. A number of flies were permitted to feed on the exudate from spontaneous cases. After ½ hour a number were introduced into a hemispherical cage applied over the right eye of a cow. Six flies were observed to walk over the lids and feed on the lacrimal fluid. After 10 minutes

[2] Budd, Laveran, and Howe, cited by Graham-Smith, G. S., Flies and disease, Cambridge, 1914, 188.

[3] Koch and others, cited by Hewitt, C. G., The house fly, Cambridge, 1914.

a number of other flies of the same lot were introduced and kept in the cage 3 or 4 minutes. The eye remained normal, diplobacilli could not be found in the lacrimal fluid 4 days later.

Experiment 3.—Sept. 25, 1923. Forty flies obtained on Sept. 24 were given a little water but no food until next morning when they were permitted to feed on the exudate from ten spontaneous cases. The flies ate ravenously. ½ hour after they had commenced to feed, twenty-one were removed. Nine were at once applied by means of a cage to the right eye of a cow. Twelve others were used 2 hours later in an attempt to infect the left eye. In no case did the flies feed on the lacrimal fluids, but several came in contact with the cornea and the lids. Four of the flies used in the second exposure were stunned or killed and placed directly on the membrana nictitans. No infection resulted to either eye.

TABLE I.

The Results of the Bacteriological Examinations of the Dejecta and External Surfaces of Flies Fed on the Exudate of Spontaneous Cases.

Lot.	No. of flies.	Examination after feeding.	No. of deposits of regurgitated or fecal material examined.	Result.	Result of examination of bottle washings.
I	6	5 min.	7	No diplobacilli.	No diplobacilli.
II	5	15 "	12	" "	Diplobacilli in moderate numbers.
III	6	1 hr.	14	" "	Diplobacilli in moderate numbers.
IV	7	2 hrs.	12	" "	No diplobacilli.
V	4	3 "	9	" "	" "

In addition to the direct attempts to transmit the virus, another series of flies was examined bacteriologically, but as a more complete observation is given in Experiment 4 the details will be omitted. It is sufficient to say that viable diplobacilli were obtained from flies 3 hours after they had been removed from the feeding bottles.

Experiment 4.—A number of recently hatched flies were obtained on Sept. 27. On Sept. 28, they were fed on the exudate from five spontaneous cases. As soon as feeding had been completed, they were removed to a series of sterile bottles. Bacteriological examinations were made of the different lots at varying intervals. In all cases isolated spots of regurgitated or fecal material were cultured separately. After this a little sterile broth was added to the bottle and the sides and bottom were rinsed. From the bottle washings plate cultures were prepared. The results are given in the appended table.

A more complete discussion of the results of this experiment will be given later. It clearly demonstrates, however, that the organism is incapable of remaining viable in the gastrointestinal tract of flies for an interval as short as 5 minutes. Furthermore it suggests that the organism may be carried on the external surfaces of flies for only relatively short periods. These facts were utilized in the later experiments.

Experiments 5 and 6.—On two occasions a number of flies were fed on the exudates of spontaneous cases. In one instance they were removed to a separate bottle in 5 minutes, in another in 15 minutes. After a second 5 and 15 minute period had elapsed, 5 cc. of bouillon was introduced into the bottles and the flies and the sides of the bottles were washed vigorously. The bouillon suspensions were divided, portions were centrifuged at high speeds, and the sediment was instilled into the left eyes of two cows. A few drops of the uncentrifuged suspension were instilled beneath the lids of the other eyes. Infection did not take place. Microscopic examination of the centrifuged sediment revealed diplobacilli in small numbers. A few characteristic colonies also appeared in the plate cultures made from the bouillon suspension.

Résumé of Attempts to Transmit the Disease with House-Flies.

Although our experiments failed to show that the disease could be transmitted by flies, it is probable that the methods are at fault. Flies are phototropic and hence gather as close to the light as possible usually at the center of the screen, the greatest distance from the eye. In no way did we find it possible to approach the natural conditions. In the experiments in which the bottle washings were used in attempts to infect, a certain number of viable bacilli must have come in contact with the eye. It is difficult to attempt to explain why the animals failed to become infected. In later experiments the eyes of both animals were inoculated with culture. One received a large dose and developed ophthalmia. The other received a small quantity of culture sprayed on the eyeball. This animal proved resistant; the organisms were able to maintain themselves in small numbers only and produced only mild symptoms. It is possible in the experiments with flies that too few organisms were introduced into relatively resistant animals, or that the organisms had been injured during the manipulation.

The more conclusive features of the experiments are those in which the excretions of flies known to have fed on material containing the bacilli were examined bacteriologically. The organism is apparently killed when ingested by the fly since it cannot be found in the regurgitated material even as soon as this material is deposited on the sides of a sterile bottle. In no instance was the organism obtained from the feces. The examinations of the bottle washings indicate that the bacilli may be carried on the external surfaces of flies for relatively short periods only. The maximum period determined thus far has been 3 hours. In another more complete experiment the organisms were viable only at the end of 1 hour.

The spontaneous disease usually reaches its highest incidence in the warm months. New cases occur, however, in the colder portions of the year. It is not probable that during cold weather the organism is transmitted by flies. It is also difficult to explain these cases by direct contact. That the virus had other means of dissemination seemed logical. Several facts were known, the bacilli were not resistant to drying, their specificity for bovines and their inability to survive 5 minutes in the digestive tract of the fly argue against an existence as a free form. Furthermore the existence of an organism so specific in character and depending for survival only on passive transmission by flies would be extremely precarious.

Further observations were made to determine whether the virus left the body in another manner. During the first epidemic the nasal exudates of a number of cows suffering from acute ophthalmia were examined. The bacillus could not be found. In November, 1923, the nasal secretions from fourteen cases were examined with negative results. In one acute case of considerable severity in a calf the organisms were encountered in the nasal passages. It was necessary to determine, if possible, whether at any stage of the disease the virus gained access to the nasal passages. With this in view three cows were inoculated in various ways and frequent bacteriological examinations were made of the eyes and nasal secretions. The protocols are given in detail.

Case 1.—On Dec. 3, 1923, a few drops of bouillon containing a portion of the surface growth from a 48 hour blood agar culture of diplobacilli were instilled beneath the lids of the left eye of a cow. ½ hour later the nasal passage in the

region of the opening of the tear duct was brushed gently with a sterile swab. Cultures were prepared from this material at once. They failed to show diplobacilli. The material about the opening of the tear duct was examined 4 and 6 hours after the inoculation with negative results. On Dec. 4, a little material was collected from the inner canthus of the left eye. It showed a moderate number of diplobacilli. Cultures were prepared from the secretion of the left nasal passage. They showed the diplobacilli present in considerable numbers. On Dec. 5, the organism was not cultivated from the nasal passages. On Dec. 6, the eye was appreciably inflamed and diplobacilli were present in the exudate. Cultures from the nostril gave negative results. Cultures were made daily during the ensuing week from the left eye and left nasal passage. The ocular reaction was pronounced and the organisms were always present. The cultures from the nasal passage were negative.

Case 2.—On Dec. 17, 1923, the growth from the slant of a 48 hour blood agar culture was suspended in 10 cc. of bouillon and by means of an atomizer a little was sprayed on the left eyeball of another cow. In addition a considerable amount was sprayed over the septum of the right nostril. 7 hours later a sterile swab was inserted into the nostrils and cultures made from the collected material. They failed to reveal diplobacilli. On Dec. 18, cultures were prepared from both eyes and both nostrils. Those from the left eye and the left nostril contained diplobacilli. The right eye and right nostril were negative. On Dec. 19, the cultures from the left eye were positive. Those from the right eye and both nostrils were negative. On Dec. 20, there occurred a visible inflammation of the left eye, the right eye was normal. The cultures from the left eye were positive. Those from the right eye and from both nostrils were again negative. On Dec. 21, the inflammation of the left eye was more severe, large numbers of diplobacilli were present in the exudate. Material obtained from the left nostril contained considerable numbers of diplobacilli. The right eye and right nostril failed to reveal the organism. No further observations were made on this animal.

Another cow treated in a similar manner proved resistant. The organisms were able to maintain themselves in the inoculated eye and produce a mild inflammation, but as far as we could determine they failed to reach the nasal passage.

The observations tend to suggest an indirect method of egress for the organism. It is apparent that in certain infections the bacilli gain access to the tear duct at least early in the course of the disease. In two of three experimental inoculations they were isolated from the nostril before symptoms of ophthalmia developed. It seems well established that their appearance in the nasal passage cannot be explained by the immediate washing down of the fluids used in the inoculations, since material from the nostrils obtained shortly after

inoculation always failed to reveal the organism. It has been also definitely established that the diplobacilli are incapable of surviving on the mucosa of the nasal passages. In spontaneous infections the organisms probably gain access to the tear duct shortly after they have come in contact with the eyes. They may even produce considerable inflammation of the duct. The irregular appearance of the bacilli in the nostril is explainable by the hypothesis that early in the disease the lumen is not sufficiently occluded with exudate and thus permits the lacrimal fluid carrying the bacilli to reach the nostril.

TABLE II.

The Effect of Treatment with Argyrol and Zinc Sulfate.

No. of cases treated.	Results of bacteriological examination of eyes before treatment.	Results of bacteriological examination of eyes after treatment.	
		3 days	*10 days*
7 with 20 per cent argyrol.	Diplobacilli in enormous numbers.	2 negative, 5 showed diplobacilli.	6 negative, 1 showed diplobacilli.
6 with 2½ per cent zinc sulfate.	Diplobacilli in enormous numbers.	None showed diplobacilli.	None showed diplobacilli.

Inasmuch as certain bacterial infections of the eye are amenable to treatment and in some earlier experiments we had succeeded in ridding the eye of the organism in certain experimental cases with a solution of zinc sulfate, we decided to test the remedial efficiency of zinc sulfate and argyrol. The cases chosen were typical infections occurring in a dairy herd. They were divided into two groups and treated by instilling the agents beneath the lids twice a day. Table II gives a brief summary of the results of the treatment. It will be noted that both drugs are of value as therapeutic agents. Zinc sulfate in the proportion of 1:40 promptly rids the eye of the bacilli.

DISCUSSION AND SUMMARY.

The experiments in which transmission of the disease was attempted by flies hardly parallel the observations within the herd. It is not unusual to observe large numbers of flies feeding on the exudate. Slight disturbances may interrupt feeding and cause the

flies to disperse and within a short period alight about the eyes of other cows.

The experiments, however, bring out the fact that the bacterium will not remain viable for even a few minutes in the digestive tract of the fly. Its life on the external surfaces of the fly is extremely short and in our observations has not exceeded 3 hours. The latter fact strengthens the opinion that in the main the infection is not dust-borne since the bacterium soon dies when not in contact with the eye.

Two other points are of considerable significance. The ability of the organism to maintain itself on the eye for considerable periods after the acute symptoms have subsided may explain the reappearance of cases during the warmer months. The organism can exist in the eye throughout the winter and with the warm weather flies may transmit it to other susceptible individuals and thus a nucleus of an epidemic may be established.

The presence of the organism in the nasal passages in the incubation stage and early in the disease in two of our experimental animals affords an explanation for the appearance of the disease in sporadic cases in the colder months. It is assumed that nasal exudate as a fine spray may be forcibly expelled and directly reach the eyeball of a normal individual. It has been shown that small quantities of culture sprayed on the cornea are capable of giving rise to the characteristic disease. The irregularity of the elimination of the organism through the nostril may be explained by the effect of inflammation on the tear duct. In experimental cases a small quantity of bouillon containing the culture was dropped or sprayed on the cornea. Doubtless the bacilli are deposited on the mucosa of the tear duct. Here they may multiply and set up an inflammation and thus gain access to the nasal passage. To what degree the virus is spread by the forcible expulsion of nasal secretion containing lacrimal fluid cannot be determined. The elimination of the bacilli from the nasal passage in our experimental inoculations leads us to believe that in the main the phenomenon is associated with early infections. The examination of the nasal passages of a large number of well established cases with negative findings tends to corroborate this view.

Of interest to those concerned with the treatment of animal diseases is the readiness with which the inflammation subsides when treated with 1:40 zinc sulfate.

THE VIABILITY OF HEMOLYTIC STREPTOCOCCUS IN CERTAIN SOLUTIONS CONTAINING GELATIN.

By FRANK L. MELENEY, M.D., and ZUNG-DAU ZAU, M.D.

(*From the Department of Surgery of Peking Union Medical College, Peking, China.*)

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INTRODUCTION.

While studying a series of cases of acute gangrene in which a hemolytic streptococcus seemed to be the causative agent, attempts were made in various ways to determine some of the special characteristics of the organism recovered. For certain quantitative biologic tests it was found to be necessary to use a suspending medium in which the streptococcus would survive without increase or diminution in numbers for a considerable length of time. We were aided by the fact that Robertson and his coworkers had recently been confronted with the same problem for the pneumococcus. After obtaining irregular results with the use of distilled water, normal saline, and Locke's solution, Robertson, Sia, and Woo demonstrated that the addition of 0.1 to 0.125 per cent gelatin greatly increased the length of life of the pneumococcus in these solutions. This pointed the way to the solution of our own problem. The series of experiments which were then carried out are reported herewith.

LITERATURE.

Falk has recently made a very careful review of the rather voluminous literature bearing upon the subject of the "influences exerted upon the vital activity of the living cell by the chemical constituents of the fluids which surround it." Loeb has contributed very largely to this literature. The most significant facts brought out by these workers, from the point of view of our problem, are these: (1) Bacteria are relatively indifferent to osmotic pressure. (2) "So far as we know there is no reason to believe that" the so called physiological solutions "are 'isotonic' as bacterial protoplasm and there is considerable evidence that such solutions do introduce cation effects which are often unwonted, commonly overlooked and probably always significant" (Falk). (3) In very dilute solutions, salts may be beneficial, but in higher concentrations the cations of univalent and

bivalent salts have a toxic or inhibitory effect on certain biologic activities of bacteria and other cells. (4) Bivalent and univalent cations antagonize each other, however, and thereby neutralize each other's toxic effect. This antagonism renders such mixtures "indifferent" or even beneficial to certain biologic activities. (5) The optimum balance between Na and Ca is about 20:1. (6) Certain of these balanced solutions may be "indifferent" to one species of bacteria and toxic for another (Zeug).

Technique.

The strain of hemolytic streptococcus used in these experiments was obtained from a case of acute superficial gangrene of the thigh, one of a series of cases previously reported (Meleney). It was a typical *Streptococcus pyogenes* in its cultured characteristics.

The organism was kept as a stock culture in horse blood broth, in which it will survive for 2 months without transfer. During the tests here to be reported it was rejuvenated by transplanting about every 7 days. For use 0.1 cc. of the horse blood broth culture was planted in 30 cc. of 1 per cent dextrose liver digest broth and incubated overnight at 37.5° C. The culture was then centrifuged and washed in the suspending fluid to be tested, in order to remove as much as possible of the culture medium and the products of bacterial metabolism. The number of organisms was estimated by the method of Gates and a standard suspension obtained containing approximately 1 billion organisms per cc. of fluid. Dilutions were then made in a manner similar to that employed by Robertson, except that larger quantities were used and the shaking time following each dilution was longer. The standard suspension was carried down through seven dilutions, each one containing a tenth of the number of organisms in the previous dilution, thus bringing the final solution down to a theoretical count of 100 cocci per cc. Enough of each dilution was made to test 1 cc. at certain time intervals. For the intervals chosen, this required 13 cc. of each. In the diluting process 30 cc. test-tubes were used and 1.5 cc. of each dilution were transferred over to 13.5 cc. of the solution in the next tube. In order to obtain as even a suspension as possible, each tube was shaken 10 minutes. Immediately after the transfer of a part for the next dilution, the rest was divided up into thirteen tubes, 1 cc. to each. While recognizing that these relatively large quantities made an even dilution more difficult, we thought that the actual error in division would be less than if smaller quantities were used. At the end of the diluting process, one series of dilutions was used to test the immediate effect of the process. 5 cc. of digest broth were added to a representative tube of each bacterial dilution and the tubes were incubated. Half of the remaining tubes were allowed to stand at room temperature, 19–21° C., and the other half at incubator temperature, 37.5°C. Then at intervals of 6, 12, 18, 24, 48, and 72 hours, broth was added to a series of dilutions, both from the room temperature group and the incubator group and these tubes were incubated.

Survival or death was then determined by a gross examination of the tubes. If grossly positive, smears were made and examined to be sure of the purity of the culture. Negative results were not recorded until after 72 hours incubation. A few tubes grew out in the second 24 hours.

Suspending Fluids.

The solutions tested were: (1) twice distilled water; (2) "normal" salt solution; (3) Locke's solution; (4) "20:1" solution; (5) 0.2 per cent sodium citrate; (6) Zeug's solution. The reason for using the first three fluids is obvious. The "20:1" solution was used on the basis of Falk's statement that the cations Na and Ca antagonized each other's toxic action in the ratio of 20:1. The solution which was used contained 1 per cent NaCl and 0.05 per cent $CaCl_2$. Sodium citrate was used in order to test a fluid in which blood would not clot and which would permit certain biologic tests with blood if found to be non-toxic for the streptococcus. Preliminary tests showed that concentrations above 0.4 per cent were slightly toxic, and therefore 0.2 per cent was used for this experiment. Zeug's solution was used to determine whether the combination and proportion of salts which he found "indifferent" for *Staphylococcus aureus* would prove to be "indifferent" also for the streptococcus. His solution contained 0.5 per cent NaCl, 0.5 per cent KCl, 0.5 per cent $CaCl_2$, and 0.1 per cent $MgCl_2$. Each fluid, both with and without 0.1 per cent gelatin, was adjusted after autoclaving to pH 7.8 with sterile M/10 NaOH or M/10 HCl, as the case might be.

Presentation of Results and Their Discussion.

Tables I to VI show the results of each fluid with and without gelatin, both at room and at incubator temperature. In every case, irrespective of the fluid used, those which contained gelatin permitted longer life than those without gelatin. Slight inconsistencies occurred in some of the tests when the organisms of a high dilution survived, while those of a lower dilution died, or when those of a certain dilution grew out at a period later than ordinary. These were probably due to a slight unevenness of dilution or to a difference in the viability of the individual chains of cocci due to the difference in their age when the original growth ceased. In the dextrose broth

the organisms grew in long chains, often consisting of 75 or more individuals and their disposition to tangle made absolutely even dilution most difficult. The inconsistencies, however, are not significant except as indicating that at those periods in which they occur,

TABLE I.

Suspending Fluid Twice Distilled Water.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution.							Temperature.	With gelatin. Period of time after dilution.						
	Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.		Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
10,000,000	+	+	+	+	+	+*	0		+	+	+	+	+	+	+
1,000,000	+	+	+	+	+	0	0		+	+	+	+	+	+	+
100,000	+	+	+	0	0	0	0	Room, 19–22°.	+	+	+	+	+	+	+
10,000	+	+	+	+	0	0	0		+	+	+	+	+	+	+
1,000	+	+	0	0	0	0	0		+	+	+	+	+	+	+
100	+	0	0	0	0	0	0		+	+	+	+	+	+*	+
100,000,000		+	+	+	+	0	0			+	+	+	+	+	0
10,000,000		+	+	+	0	0	0			+	+	+	+	0	0
1,000,000		+	+	+	+	0	0	Incubator, 37.5°.		+	+	+	+	+	0
100,000		+	+	+	0	0	0			+	0	0	0	0	0
10,000		0	0	0	0	0	0			+	+	+	0	0	0
1,000		0	0	0	0	0	0			+	+	0	0	0	0
100		0	0	0	0	0	0			+	0	0	0	0	0

5 cc. of digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

*Grew out in the second 24 hours; probably considerable destruction of bacteria.

there was considerable destruction of the cocci even in those tubes which grew out.

These tables seem to demonstrate definitely a protection of some kind on the part of the gelatin, and yet it is seen that the protection is not an absolute one and that the various gelatin solutions are of

varying efficiency in their protective action. This suggests that the gelatin is used up in the course of time or that it forms a coating for the organisms which only delays the penetration of the salts or water in toxic or lytic quantities. In the gelatin solutions, the death of

TABLE II.

Suspending Fluid 0.9 Per Cent NaCl.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution.							Temperature.	With gelatin. Period of time after dilution.						
	Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.		Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	0	0	0	0	0	0		+	+	+	+	+	0	0
10,000,000	0	0	0	0	0	0	0		+	+	+	+	+	0	0
1,000,000	0	0	0	0	0	0	0	Room, 19–22°.	+	+	+	+	0	0	0
100,000	0	0	0	0	0	0	0		+	+	+	+	0	0	0
10,000	0	0	0	0	0	0	0		+	+	0	+	0	0	0
1,000	0	0	0	0	0	0	0		+	0	0	0	0	0	0
100	0	0	0	0	0	0	0		0	0	0	0	0	0	0
100,000,000		0	0	0	0	0	0			+	0	0	0	0	0
10,000,000		0	0	0	0	0	0			0	0	0	0	0	0
1,000,000		0	0	0	0	0	0	Incubator, 37.5°.		+	0	0	0	0	0
100,000		0	0	0	0	0	0			0	0	0	0	0	0
10,000		0	0	0	0	0	0			0	0	0	0	0	0
1,000		0	0	0	0	0	0			0	0	0	0	0	0
100		0	0	0	0	0	0			0	0	0	0	0	0

5 cc. of digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

the organisms, although considerably delayed, takes place in much the same order as in the solutions without gelatin. One cannot draw strictly accurate conclusions regarding the relative efficiency of the different solutions from Tables I to VI because of the slight variation of conditions which tests are necessarily subjected to when

performed on different days. In order to compare them properly with each other, all six gelatin solutions had to be tested at the same time.

TABLE III.

Suspending Fluid Locke's Solution.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution. Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.	Temperature.	With gelatin. Period of time after dilution. Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
10,000,000	+	+	+	+	+	+	+*		+	+	+	+	+	+	+
1,000,000	+	+	+	+	+	+	0		+	+	+	+	+	+	+
100,000	+	+	+	+	+	+	+*	Room, 19–22°.	+	+	+	+	+	+	+
10,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
1,000	+	+	+	+*	+	0	0		+	+	+	+	+	+	+
100	+	0	+	0	+*	0	0		+	+	+	+	+	+	+
100,000,000		+	+	+	0	0	0			+	+	+	+*	0	0
10,000,000		+	0	0	0	0	0			+	+	+	0	0	0
1,000,000		0	0	0	0	0	0	Incubator, 37.5°.		+	+	+	+*	+	0
100,000		0	0	0	0	0	0			+	+	+	0	0	0
10,000		0	0	0	0	0	0			+	+	+	0	0	0
1,000		0	0	0	0	0	0			+	+	0	0	0	0
100		0	0	0	0	0	0			+	+	0	0	0	0

5 cc. digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

* Grew out in the second 24 hours; probably considerable destruction of bacteria.

For this purpose the sixth and seventh dilutions, estimated to contain respectively 1,000 and 100 cocci per cc., were used. The tubes, containing 1 cc. quantities of these dilutions, were kept at room temperature and 5 cc. of digest broth were added to each tube at the proper time intervals. One test was made immediately after the dilution process, others at 12 and 24 hours and thereafter daily for 15 days. The result of this test is shown in Table VII.

The table indicates an early death in the gelatin-saline, a somewhat later death in gelatin-water and gelatin-Zeug's mixture, and a fairly long maintenance of life in gelatin-"20:1," gelatin-Locke's,

TABLE IV.

Suspending Fluid "20:1" Solution.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution.							Temperature.	With gelatin. Period of time after dilution.						
	Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.		Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	+	+	+	+	+	+*		+	+	+	+	+	+	+
10,000,000	+	+	+	+	+	0	+*		+	+	+	+	+	+	+
1,000,000	+	+	+	+	+*	+	0		+	+	+	+	+	+	+
100,000	+	+	+	+	0	0	0	Room, 19–22°.	+	+	+	+	+	+	+
10,000	+	+	+	0	0	0	0		+	+	+	+	+	+	+
1,000	+	+	+	+	0	0	0		+	+	+	+	+	+	+
100	+	+	0	0	0	0	0		+	+	+	+	+	+	+
100,000,000		+	+	+	+*	0	0			+	+	+	0	+	0
10,000,000		+	+	+	+	0	0			+	+	+	0	0	0
1,000,000		+	+	+	+*	0	0			+	+	+	0	0	0
100,000		+	+	+	0	0	0	Incubator, 37.5°.		+	+	+	0	0	+
10,000		0	0	0	0	0	0			+	+	+	+	0	0
1,000		0	0	0	0	0	0			+	+	+	0	0	0
100		0	0	0	0	0	0			+	+	+	0	0	0

5 cc. of digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

* Grew out in the second 24 hours; probably considerable destruction of bacteria.

and gelatin-citrate. Thus it is seen that three of these six solutions are very efficient in maintaining the streptococcus. In them it may be depended upon to live in very high dilutions for a week or more at room temperature or for 12 hours at incubator temperature.

The beneficial action of the sodium citrate solution is very striking. In some manner the toxic action of the sodium is inhibited. Here is a non-toxic solution with a single cation. It may be non-toxic

TABLE V.

Suspending Fluid 0.2 Per Cent Sodium Citrate Solution.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution.							Temperature.	With gelatin. Period of time after dilution.						
	Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.		Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
10,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
1,000,000	+	+	+	+	+*	+*	+*		+	+	+	+	+	+	+
100,000	+	+	+	+	+*	+*	+	Room, 19–22°.	+	+	+	+	+	+	+
10,000	+	+	+	+	+*	+*	+*		+	+	+	+	+	+	+
1,000	+	+	+	0	+*	0	0		+	+	+	+	+	+	+
100	+	+	+	0	0	0	+*		+	+	+	+	+	+	+*
100,000,000		+	+	0	+*	0	0			+	+	+	+*	+*	0
10,000,000		+	+	0	0	0	0			+	+	0	0	0	0
1,000,000		+	0	0	0	0	0			+	+	+	+	0	0
100,000		0	0	0	0	0	0	Incubator, 37.5°.		+	+	+	+*	0	0
10,000		0	0	0	0	0	0			+	+	+	+	0	0
1,000		0	0	0	0	0	0			+	+	+	0	0	0
100		0	0	0	0	0	0			+	+	0	+*	0	0

5 cc. of digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

* Grew out in the second 24 hours; probably considerable destruction of bacteria.

because it is relatively dilute, for, as stated above, higher concentrations were found to be toxic. However, when sodium chloride is diluted down to approximately the same sodium ion concentration it is found to be less efficient than the citrate solution.

An experiment was carried out with 0.2 per cent sodium citrate and 0.097 per cent sodium chloride solutions, each containing 0.1 per cent gelatin. The solutions were titrated after autoclaving to pH 7.8. The test was similar to the preceding one in the manner of dilution and time intervals of culture. The tubes were kept at room temperature.

TABLE VI.

Suspending Fluid Zeug's Mixture.

Estimated No. of cocci per cc.	Without gelatin. Period of time after dilution. Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.	Temperature.	With gelatin. Period of time after dilution. Immediately.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.
100,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
10,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
1,000,000	+	+	+	+	+	+	+		+	+	+	+	+	+	+
100,000	+	+	+	+	0	0	0	Room, 19–22°.	+	+	+	+	+	+	+
10,000	+	+	+	+	0	0	0		+	+	+	+	+	+*	+
1,000	+	0	0	0	0	0	0		+	+	+	+	+	+*	+
100	+	0	0	0	0	0	0		+	+	+	+	+	+*	0
100,000,000		+	+	+	+	0	0			+	+	+	+	+*	0
10,000,000		+	0	0	0	0	0			+	+	+	+	0	0
1,000,000		0	0	0	0	0	0			+	+	+	+	0	0
100,000		0	0	0	0	0	0	Incubator, 37.5°.		+	+	+	0	0	0
10,000		0	0	0	0	0	0			+	+	+	0	0	0
1,000		0	0	0	0	0	0			+	0	0	0	0	0
100		0	0	0	0	0	0			+	0	0	0	0	0

5 cc. of digest broth pH 7.6 were added to each tube at the intervals indicated. The tubes were then incubated at 37.5°.

+ represents growth in the broth tubes.

0 represents no growth. Negative results were not recorded until after 72 hours incubation.

* Grew out in the second 24 hours; probably considerable destruction of bacteria.

It was found that the sixth and seventh dilutions of the sodium chloride suspension maintained life for 8 and 7 days respectively. In both citrate dilutions, however, the streptococci were preserved for 12 days. Thus it is seen that although the dilute saline is less

toxic then "normal" saline, it is still inferior to the citrate as a preserving fluid. In order to rule out an alteration in hydrogen ion

TABLE VII.

A Comparison of All Six Gelatin Solutions under Identical Conditions.

Dilution No.	No. of cocci per cc.	Twice distilled water.	0.9 per cent sodium chloride.	Locke's solution.	"20:1" solution.	0.2 per cent sodium citrate.	Zeug's solution.
6	1,000	7	4	15+	10	15+	6
7	100	4	2	8	12	15+	7

The figures indicate the last survival day for each of the sixth and seventh dilutions of the solutions indicated. Toward the end there were some irregularities in each case so that in two of the solutions the seventh dilution grew out once after the sixth dilution had succumbed. The test was carried out for only 15 days. Therefore the limit may not have been reached for the citrate and for the sixth dilution of Locke's.

TABLE VIII.

Varying Effects of Simple Rotation and Violent Agitation in Making Dilutions.

Solution.	Method of mixture.	Estimated No. of cocci per cc.	Period of time after dilution.						
			Immediately.	1 hr.	3 hrs.	6 hrs.	15 hrs.	24 hrs.	48 hrs.
Locke's with gelatin.	Rotation.	1,000	44	33	52	48	44	47	2
	Agitation.	1,000	—*	140	130	110	85	95	43
	Rotation	100	8	7	8	3	5	2	1
	Agitation.	100	16	13	19	18	11	2	3
0.2 per cent sodium citrate with gelatin.	Rotation.	1,000	180	215	230	210	185	145	110
	Agitation	1,000	740	760	705	640	450	430	285
	Rotation.	100	15	19	26	20	15	14	8
	Agitation.	100	45	32	50	37	46	50	19

The figures represent the number of colonies on the agar plates. The number is considerably higher in the citrate than in the Locke's solution test. This may be due to a more effective breaking up of the chains as well as to a better protection for the less viable cocci.

* Killed by hot agar.

concentration as the cause of the difference between the two solutions, the final hydrogen ion concentration was determined on the 14th

day of the test. It was found that that of the citrate solution had risen to pH 8.2 while that of the salt solution had fallen to pH 7.4. Each solution had varied from the original figure to the same degree but in an opposite direction. It is possible that this may have had some effect, but, as is pointed out below, both figures are within the zone of hydrogen ion concentration at which the streptococcus may be expected to survive. It would seem then that the difference between the saline and the citrate solutions is not due to the concentration of sodium ions but may be due in part to the difference in the anions present. Anion effect has been recorded by a number of observers, and Falk has made the statement that the anion may be nutritive, particularly if it contain carbon.

There is evidence also that the gelatin may protect the organisms against the mechanical injury of the dilution process, as was observed with the pneumococcus (Robertson, Sia, and Woo). This is shown in an experiment in which the dilution process was performed in two different ways.

In one, dilutions were made in 10 cc. of fluid gently rotated in flasks; in the other, 2 cc. of fluid were violently agitated in test-tubes. Locke's solution, 0.2 per cent sodium citrate, and the "20:1" solution were all tested without gelatin. In each case the standard suspensions were carried down through eight dilutions and 1 cc. of each dilution was plated with digest agar, both in the rotation and in the agitation series. After incubation the colonies were counted. In each instance the colonies in the rotation series were more numerous than in the agitation series and the streptococci were preserved in a higher dilution by the former process of dilution. When a similar experiment was carried out with gelatin solutions there were found to be more colonies in the agitation series than in the rotation series. Gelatin-Locke's and gelatin-citrate solutions were so tested. Owing to the greater preservation in the gelatin solutions, 1 cc. quantities of the sixth and seventh dilutions were plated at intervals of 1, 3, 6, 15, 24, and 48 hours.

The results are shown in Table VIII. Apparently, in the gelatin solution, the agitation merely serves to break up the chains into smaller fragments so that more colonies are produced when the plates are poured. The higher counts in the citrate solution may indicate that an increased degree of agitation has broken up the chains better, or that the citrate affords a better preservation for the less viable cocci.

Table VIII further shows that in the 0.1 per cent gelatin solutions the number of colonies is maintained at a fairly constant level for from 15 to 24 hours, but after that the number gradually falls off. This tends to substantiate the theory that the gelatin is used up; as is suggested above. It would be used up, of course, if it were utilized by the organisms as a nutrient substance. An experiment was done with 0.5 and 1.0 per cent concentrations of gelatin in Locke's solution to determine the point at which actual multiplication would take place. Dilutions were made in the usual way and the sixth

TABLE IX.

Locke's Solution with Varying Concentrations of Gelatin.

Gelatin.	Estimated No. of cocci per cc.	Room temperature, 19–21°. Period of time after dilution.							
		Immediately.	1 hr.	3 hrs.	6 hrs.	15 hrs.	24 hrs.	48 hrs.	72 hrs.
per cent									
0.5	1,000	170	220	150	145	130	245	2,500	2,800
	100	14	23	21	11	15	17	100	70
1.0	1,000	235	250	100	145	265	1,100	20,000	40,000
	100	36	33	20	20	30	110	2,000	5,000

The figures represent the number of colonies on the agar plates. It will be seen that in the 1 per cent gelatin solution there is a marked growth in the last part of the first 24 hours. In the 0.5 per cent gelatin there is a growth in the second 24 hours.

and seventh dilutions were plated at intervals of 1, 3, 6, 15, 24, 48, and 72 hours. The tubes were kept at room temperature. It was found that there was slight multiplication after 15 hours in the 0.5 per cent gelatin tubes and marked growth in the 1.0 per cent gelatin. Table IX gives the figures for this experiment. Gelatin is found to be nutrient, therefore, in the higher concentrations, and it seems reasonable to conclude that it is also slightly nutrient in the concentration of 0.1 per cent, at least sufficiently so to prevent autolysis or to permit a slow metabolism without multiplication. If reproduction does take place it is offset by the death of other individuals.

In order to determine the zone of hydrogen ion concentration in which the streptococcus could survive in gelatin solutions throughout the period of our tests, gelatin-water, gelatin-Locke's, and gelatin-citrate solutions were tested.

In each case the fluids were adjusted after sterilization to pH values ranging from 4.8 to 9.6 at intervals of 0.6 and were kept in the ice box overnight to stabilize the reaction. The acid range was obtained by adding M/10 HCl and the alkaline range by M/10 NaOH. The solutions were used for washing and diluting in the usual way and cultures were made of the sixth and seventh dilutions at the time intervals selected for the original tests. At 24 hour intervals the hydrogen ion concentration of each fluid was again estimated.

With the gelatin-Locke's solution no buffer was used other than the small amount of sodium bicarbonate in the solution. The original reaction of the solution was pH 7.5. At the end of the dilution process it was found that the two highest alkaline levels had fallen markedly, possibly owing to the absorption of CO_2 from the air, while the acid ranges remained fairly constant. The highest alkaline level which could be maintained throughout the test was pH 8.2. The organisms survived in all of the tubes throughout the dilution process, but in the first 6 hours they gradually died off in the acid tubes. After 72 hours, death had occurred in all of the tubes containing fluid with a reaction of pH 4.8, 5.4, and 6.0, and in the seventh dilution of pH 6.6. In all of the other tubes the streptococci survived for 3 days. The final survival zone of the highest dilution lay between pH 7.4 and 8.2 although for a time at least these surviving organisms had been in contact with alkaline fluid ranging from pH 7.2 to 9.6.

The original reaction of the gelatin-water was pH 5.6. After titrating to the pH levels desired, 1 per cent of Clark's standard buffer solutions was added. In spite of the buffer, the hydrogen ion concentration of all of the solutions above pH 6.0 fell several points during the dilution process,—the highest falling markedly. More alkali was then added to bring the solutions again to the desired levels and tests were made immediately and at the usual time intervals. After 24 hours it was found that the hydrogen ion concentration of the higher ranges had fallen again. It was also evident that there was a delay in the growth of the seventh dilutions of the pH 6.0,

6.6, and 9.6 tubes. At the end of 72 hours, however, it was found that the streptococci had survived in the highest dilution through a zone ranging from pH 6.1 to 8.4. pH 8.4 was the highest level which could be maintained throughout the test.

The original reaction of the gelatin-citrate was pH 7.2. After titrating to the pH levels desired, 2 per cent of the standard buffer solutions was added. In this experiment also, the highest alkaline levels fell during the dilution process. After 72 hours it was found that the streptococci had survived in the highest dilution through a zone ranging from pH 6.2 to 8.2; pH 8.2 was the highest level which could be maintained throughout the test. These tests indicate that there is a fairly wide zone of hydrogen ion concentration in which the organisms will survive in the gelatin containing solutions for at least 3 days. The upper limit has not been determined but if the original solutions are titrated to pH 7.8 to 8.0, the level will be maintained within the zone in which the streptococci may be expected to survive for a considerable length of time.

CONCLUSIONS.

1. The findings of Robertson, Sia, and Woo with regard to the preservative action of 0.1 per cent gelatin in suspending fluids for the pneumococcus have been confirmed for the streptococcus.

2. In gelatin-citrate, gelatin-Locke's, and gelatin-"20:1" solutions, the streptococcus will live for 3 days or longer at room temperature and for 12 hours or longer at incubator temperature in dilutions as high as 100 cocci per cc.

3. The gelatin in 0.1 per cent concentration maintains life at a fairly constant numerical level for from 15 to 24 hours. These fluids may therefore be used for certain quantitative biologic tests.

4. In slightly higher concentrations of gelatin, there is actual growth which suggests that even in 0.1 per cent concentrations the gelatin has a nutrient action.

5. There is also evidence that gelatin affords some protection against the mechanical injury of the dilution process.

6. The toxic action of unbalanced salts, the possible lytic action of water, and the autolytic action of the organisms themselves are all

delayed by the presence of gelatin, but eventually these actions take place in the same manner that has been described before by many observers and as here observed for solutions without gelatin.

7. The life of streptococci is maintained in these gelatin solutions through a relatively wide zone of hydrogen ion concentration.

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AN HISTOLOGICAL STUDY OF THE CENTRAL NERVOUS SYSTEM IN EXPERIMENTAL BOTULINUS POISONING.

By E. V. COWDRY and F. M. NICHOLSON.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Recent physiological experiments by Dickson and Shevky[1] and by Edmunds and Long[2] afford evidence that the peculiar and distressing symptoms of *botulinus* poisoning may be explained upon the hypothesis that the real site of action of the toxin is upon nerve terminals and that the paralytic features are not of central origin, as was formerly supposed. This casts a measure of suspicion upon the older work, largely histological, tending to show that the toxin acts directly upon the central nervous system and particularly upon the respiratory center presumably in the medulla. If the position taken by these authors is as secure as it seems to be, how then are we to explain the repeated descriptions in the literature of definite lesions with claims that they result from poisoning by *botulinus* toxin? Are they, perhaps, susceptible of some other interpretation, or is their relationship to botulism a fact which can be substantiated? A brief review of the literature will be helpful in showing how contradictory are the observations that have been made.

Van Ermengem's [3] discovery that the toxin is produced by the growth of *Clostridium botulinum*, coming at a time when so many and brilliant advances were being made in the field of neurocytology by Golgi, Cajal, Nissl, and their students, led many investigators to approach the problem from this angle, and to attribute considerable, perhaps undue, significance to the results obtained. The observation, soon reported, of chromatolytic changes in the Nissl bodies was responsible in the first instance, for the claim that the toxin acts primarily upon nerve cells and that all other manifestations of disease are secondary phenomena. But there has been no general agreement as to the distribution of the lesions. Some con-

[1] Dickson, E. C., and Shevky, R., *J. Exp. Med.*, 1923, xxxvii; 711, xxxviii, 327.

[2] Edmunds, C. W., and Long, P. H., *J. Am. Med. Assn.*, 1923, lxxxi, 542.

[3] Van Ermengem, E., *Arch. pharmacod.*, 1897, iii, 213.

sidered them to be most marked in the spinal cord and to decrease in severity as the higher centers are reached, [4,5] while others emphasized particularly the changes in the nuclei of origin of the cranial nerves, not even looking for lesions elsewhere.[6,7] According to Pisani,[8] the pathological changes, though occurring in other places, are most marked in the parasympathetic system. That the clinical symptoms are not always what one would anticipate from the topography of the lesions has been pointed out by Wilbur and Ophüls.[9] Certainly one would not expect noticeable involvement of the cells of the cerebral and cerebellar cortex. Neither is the severity of the lesions paralleled by the clinical symptoms.[10] Further uncertainty is cast on the findings by the failure of Wilbur and Ophüls to detect any definite alterations at all in nerve cells and by their willingness to go so far in the opposite direction as to claim that the Nissl bodies stain unusually well. Dickson, [11] also, was unable to bring to light distinctive changes in nerve cells. Some additional weakness is revealed upon critical examination of the evidence presented in favor of the actual existence of lesions within the cells of the brain and spinal cord. For instance, none of the papers is provided with good enough illustrations for us to ascertain with any degree of accuracy the real nature of the alterations observed. It is likewise to be regretted that there are no available records supplying quantitative data of the percentage of nerve cells altered and the exact degree of change, so that the personal equation of interpretation is a large one in each instance. For this reason it may be questioned whether some of the alleged modifications do not fall within the normal range of variation. In studying a problem so important it is surprising that so little has been done with well controlled experimental animals and that so many investigators have contented themselves with autopsy findings.

Turning now to the vascular system we find that, though the majority of investigators has reported marked congestion of the blood vessels with small hemorrhages into the brain substance, and Dickson has felt justified in concluding that thrombosis of the type observed by Ophüls is pathognomonic of botulism, there are still many points which remain to be settled. Leucocytic perivascular infiltrations have only been described by some authors and there seems to be a lack of agreement as to the type of cell which predominates. It is characterized as lymphocytic by Semerak,[7] and Paulus[12] speaks of the infiltration as consisting of

[4] Marinesco, G., *Presse méd.*, 1897, v, 41.

[5] Ossipoff, V. P., *Ann. Inst. Pasteur*, 1900, xiv, 769.

[6] Römer, P., and Stein, L., *Arch. Ophth.*, 1904, lviii, 291.

[7] Semerak, C. B., *J. Infect. Dis.*, 1921, xxix, 190.

[8] Pisani, S., *Policlinico, sez. med.*, 1922, xxix, 567.

[9] Wilbur, R. L.,and Ophüls, W., *Arch. Int. Med.*, 1914, xiv, 589.

[10] Kempner, W., and Pollack, B., *Deutsch. med. Woch.*, 1897, xxiii, 505.

[11] Dickson, E. C., Botulism. A clinical and experimental study, Monograph of The Rockefeller Institute for Medical Research, No. 8, New York, 1918.

[12] Paulus, E. A., *J. Psychol. u. Neurol.* 1914, xxi, 201.

lymphocytes and plasma cells, while Warthin [13] has referred to the "infiltration of polymorphonuclears with some lymphocytes." Judging from published accounts, edema may be very pronounced [14] or not sufficiently marked to attract attention.[15] One author has mentioned the occurrence of neurophagocytosis [5] and another the formation of gas bubbles within the blood vessels and in the brain substance.[13] Variability in the reaction of different laboratory animals has evidently been also a confusing factor.

In view of these considerations we have made a reexamination of the changes in the nervous system, detectable microscopically, in animals suffering from *botulinus* poisoning, in order to ascertain, as precisely as possible, the exact degree of involvement of the brain and spinal cord and in this way to learn whether the histological evidence is actually as incompatible with the physiological developments as, judging from the literature, it might seem to be. We have been greatly aided by the kindness of Dr. Bronfenbrenner in giving us animals in every desired stage of reaction to the toxin and for many helpful conferences in the course of the work.

Technique.

After some preliminary experiments with methylene blue, toluidine blue, and other conventional stains for Nissl bodies in alcohol-fixed tissues, we found that better and more uniform results could be obtained by simply coloring with Giemsa's stain material preserved in Zenker's fluid (without acetic acid). The technique is given in detail by Wolbach.[16] This method has the advantage of yielding a good contrast coloration with the red component (Azur II eosin) and at the same time of serving well for the detection of microorganisms and for the routine study of the lesions. In several experiments animals were colored vitally with trypan blue in the hope of locating injured areas by deposition of the dye. Sections were cut in series and as many were mounted on each slide as could be fitted under a 22 by 60 mm. cover-glass.

Summary of Experiments.

Healthy mice, guinea pigs, and rabbits selected by Dr. Bronfenbrenner were injected with the toxin and kept by him until they showed

[13] Warthin, A. S., *Tr. Assn. Am. Phys.*, 1922, xxxvii, 357.
[14] Brosch, A., *Wien. klin. Woch.*, 1896, ix, 219.
[15] Nevin, M., *J. Infect Dis.*, 1921, xxviii, 226.
[16] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 185.

very pronounced symptoms, when we etherized them and removed the tissues for microscopic examination. The toxin was obtained by the growth of *Bacillus botulinus* upon mince meat broth and was filtered about 2 months previously. The M.L.D. producing death of a mouse of 17 to 21 gm. within 48 hours was 0.000005 cc.; the M.L.D. for a guinea pig of 350 to 400 gm., 0.00004 cc.; and for a rabbit of 1,700 gm., 0.0002 cc. By using animals of different weights and employing different dosage of toxin a graded series of reactions of from 1 to 9 days duration was obtained. Abundant controls were supplied by our experiments upon encephalitis in rabbits[17] and in mice.[18] For convenience we have set forth very briefly the data concerning the material employed as follows:

Mice.

Toxin + Trypan Blue:

Mouse 1. M.L.D. intraperitoneally + 0.4 cc. 1 per cent trypan blue subcutaneously; died in 48 hours. Perfused with salt solution followed by 20 per cent formalin. Complete serial sections, 10 microns, anterior commissure to C 1, counterstained with alum-carmine. 31 slides.

Mouse 2. 0.000004 cc. toxin + 0.4 cc. trypan blue; after 48 hours 0.5 cc. more trypan blue; after 72 hours 0.005 cc. toxin + 0.5 cc. additional trypan blue; after 80 hours etherized. Sections, as above, infundibulum to C 1. 25 slides.

Mouse 3. 0.000004 cc. toxin + 0.4 cc. trypan blue; after 48 hours 0.5 cc. more trypan blue; after 72 hours etherized. Sections as in Mouse 2. 34 slides.

Mouse 4. M.L.D., otherwise same as Mouse 3. Sections anterior border septum pellucidum to C 1. 26 slides.

Toxin Alone:

Mouse 5. 0.00001 cc. toxin; after 17¼ hours died; fixed immediately in 95 per cent alcohol diluted from absolute. Complete serial sections, 7.5 microns, posterior commissure to C 1. Giemsa. 18 slides.

Mouse 6. 0.0001 cc. toxin; after 24 hours died; fixed in Zenker (less acetic). Sections same as Mouse 5. 18 slides.

Mouse 7. 0.0001 cc. toxin, same as Mouse 6. 22 slides.

Mouse 8. 0.00001 cc. toxin; after 20 hours died; otherwise same as Mouse 5. 21 slides.

Mouse 9. 0.00001 cc. toxin; after 20½ hours died; otherwise same as Mouse 5. 21 slides.

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[17] Cowdry, E. V., and Nicholson, F. M., *J. Exp. Med.*, 1923, xxxviii, 695.

[18] Cowdry, E. V., and Nicholson, F. M., *J. Exp. Med.*, 1924, xl (in press).

Mouse 10. 0.000005 cc. toxin; after 3 days died; fixed tissues in formalin, ammoniated alcohol, and Marchi for nerve fibers.

Normal Controls:[19]

Mice 11 to 82, including mice of different ages.

Specificity Controls:[19]

Mice 83 to 94. Injected with Kieselgur, starch, carmine, India ink, trypan blue, manganese dioxide, and mercuric chloride.

Mice 95 to 101. Mouse typhoid-immune animals.

Mice 102 to 107 with large transplantable tumors.

Mice 108 to 113. Injected with emulsion of kidneys of tumor mice.

Guinea Pigs.

Toxin Alone:

Guinea Pig 1. 0.00005 cc. toxin subcutaneously; after 71 hours etherized. Serial sections through midbrain, at two levels in medulla and at C 1. Zenker-Giemsa. 27 slides.

Guinea Pig 2. 0.000025 cc. toxin; after 69 hours etherized. 95 per cent alcohol, Giemsa, serial sections through midbrain, medulla, and C 1. 37 slides.

Guinea Pig 3. M.L.D.; after 48 hours etherized. 95 per cent alcohol, Giemsa, serial sections through brain stem at 4 levels. 34 slides.

Guinea Pig 4. M.L.D.; after 4 days etherized. 95 per cent alcohol, Giemsa, serial sections through brain stem (but many lost). 41 slides.

Guinea Pig 5. M.L.D.; after 4 days etherized. 95 per cent alcohol, Giemsa, serial sections taking alternate rows of 6 to 10 sections midbrain to C 1. 42 slides.

Guinea Pig 6. M.L.D., after 4 days etherized. Same. 40 slides.

Guinea Pig 7. M.L.D., after 8 days died. Same technique. 52 slides.

Guinea Pig 8. M.L.D.; after 6 days etherized. Same technique. 40 slides.

Guinea Pig 9. M.L.D. Same. 48 slides.

Guinea Pig 10. 0.00005 cc. toxin; after 6 days received antitoxin and died. Same technique. 3 slides.

Guinea Pig 11. M.L.D.; after 7 days etherized. Fixed partly in 95 per cent alcohol and partly in Regaud's fluid (for mitochondria).

Guinea Pig 12. M.L.D.; after 9 days etherized. 95 per cent alcohol, Giemsa.

Guinea Pig 13. M.L.D.; after 9 days etherized. Zenker (less acetic), serial sections of brain at level of septum pellucidum, infundibulum, inferior olive, lower part of medulla, and spinal cord. 20 slides.

Guinea Pig 14. M.L.D.; after 7 days etherized. Same technique. 10 levels. 10 slides.

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[19] Some of these brains were cut into complete serial sections, while in the case of the remainder a few serial sections were taken at the following levels: olfactory bulbs, optic chiasma, corpora mammillaria, corpus trapezoideum, and decussation of the pyramids.

Guinea Pig 15. M.L.D.; after 7 days etherized. Same technique. 13 levels. 13 slides.

Guinea Pig 16. M.L.D.; after 7 days etherized. Same technique. 10 levels. 10 slides.

Guinea Pig 17. 0.2 cc. refiltered toxin heated; 4 days etherized. Same technique. 8 levels. 8 slides.

Guinea Pig 18. M.L.D.; refiltered toxin; 4 days etherized. Same technique. 7 levels. 7 slides.

Guinea Pig 19. M.L.D.; refiltered toxin; 4 days etherized. Same technique. 5 levels. 5 slides.

Guinea Pig 20. 0.075 cc. toxin; died after 12 hours. Fixed tissues in Zenker with acetic, Rubaschkin's mixture, and da Fano's pyridine mixture for neuroglia.

Specificity Controls:

Guinea Pigs 21 to 23. Injected intraperitoneally with kidney emulsion from Mice 17 to 22. Zenker (less acetic), Giemsa, several sections at different levels.

Guinea Pigs 24 to 37. Infected with typhus fever, same technique, several sections.

Rabbits.

Toxin Alone:

Rabbit 1. M.L.D. intravenously; after 48 hours etherized. 95 per cent alcohol, Giemsa. Serial sections, alternate rows from near rostral border of posterior commissure to C 1. 94 slides.

Rabbit 2. M.L.D. + 0.0004 cc. antitoxin; after 48 hours etherized. Same technique, from caudal border of optic chiasma to caudal margin of inferior olive. 45 slides.

Rabbit 3. M.L.D. Zenker (less acetic), Giemsa. Serial sections at 4 levels. 9 slides.

Normal Controls:

Rabbits 4 to 9. Apparently normal.

Rabbits 10 to 15. Protected carefully against snuffles infection.

Specificity Controls:

Rabbits 16 to 20. Subcutaneous injections of benzene in olive oil.

Rabbits 21 to 48. Experimental herpetic encephalitis.[17]

Rabbit 50. Injected with mouse kidney emulsion (Mice 17 to 22).

Rabbit 51. Discarded from animal room owing to sickness (torticollis).

OBSERVATIONS.

Nerve Cells.—A study carried on for several years by one of us[20] of chromatolytic changes in rats following ligation of the hypoglossal

[20] Nicholson, F. M., *J. Comp. Neurol.*, 1923, xxxvi, 37.

nerve has been helpful in our attempt to detect changes in the Nissl bodies. In this search attention was concentrated upon the brain stem (including the respiratory centers) and the cervical cord, but a number of complete sets of serial sections through entire brains was also thoroughly examined. Doubtful cases were at once referred to these standardized rat preparations, embracing slides from 294 animals; but although a very systematic study was made, section by section, with the aid of a mechanical stage, of the brains of mice, guinea pigs, and rabbits poisoned with *botulinus* toxin no alterations were found in the Nissl bodies or in the nuclei of the nerve cells which were not likewise observed in apparently normal controls or which were not obviously produced by traumatism in the removal of the brains. From this we assumed that an axone reaction, if it exists, is but slight.

Mitochondria were not extensively studied because the indications are that morphological and tinctorial changes in these elements, detectable by our present methods of technique, are not readily induced experimentally in nerve cells;[21-23] but a few sections (of Guinea Pig 11) were stained with fuchsin and methyl green[24] without revealing anything in the way of distinctive changes. The Golgi apparatus was not studied for the reason that the technique for its demonstration is not adapted to the survey of large amounts of tissue. Neither were preparations made designed to show the neurofibrils.[25]

Nerve Fibers.—On the chance that some injury might have occurred, insufficient to bring about a noticeable axone reaction within the cells but which might nevertheless be located by the direct examination of cell processes, several preparations were made (of Mouse

[21] Clark, E., *J. Comp. Neurol.*, 1914, xxiv, 61.

[22] Strongman, B. T., *Anat. Rec.*, 1917, xii, 167.

[23] McCann, G. F., *J. Exp. Med.*, 1918, xxvii, 31.

[24] Cowdry, E. V., Carnegie Institution of Washington, Pub. No. 11, Contributions to Embryology, 1916, iv, 27.

[25] It was recognized that the interneuronal synapses are important elements which must be borne in mind in attempts to explain the symptoms of poisoning; but they escape histologic enquiry except perhaps in rare cases in certain teleosts (Bartelmez, G. W., *J. Comp. Neurol.*, 1915, xxv, 87).

10) by the methods of Weigert,[26] Marchi,[26] and Ranson[27] but without result. Either the fibers were unmodified or else they were injured so slightly as to escape completely detection by microscopic examination.

Neuroglia Cells and Fibers.—Although the clinical symptoms were too acute to be explained solely on the basis of involvement of the neuroglia, a few special preparations were made to supplement our regular examination of Giemsa specimens. This was done in consideration of the fact that the recent discoveries of Rio Hortega[28] and the Spanish school of neurologists regarding the participation of neuroglia in pathologic changes could hardly have been predicted in advance. The method advised by Rubaschkin[29] and said to be particularly useful in the case of the smaller mammals was applied (to Guinea Pig 20) with disappointing results. Neither did we obtain good preparations by da Fano's[30] method.

Obviously here, as well as in the case of the mitochondria and the nerve fibers, our observations were inconclusive because the special technique necessary was not applied to a sufficient number of animals.

Vascular Engorgement, Thrombosis, Hemorrhage, and Edema.—A slight degree of congestion of the blood vessels of the meninges and at the base of the brain was our only fairly constant pathological finding. It was noted in all three species of animals studied. It varied in degree, being noticeable macroscopically in about 75 per cent. On the other hand, a few animals failed to show congestion even upon detailed histological examination.

Thrombosis was not observed except occasionally in the meningeal blood vessels of four guinea pigs (Guinea Pigs 2, 5 to 7) in which it was susceptible of explanation on the basis of a mild meningitis to be referred to in the following paragraphs.

Small hemorrhages were noted in a few mice but they were not sufficiently marked to exclude the possibility of their having been

[26] Mallory, F. B., and Wright, J. H., Pathological technique, 7th edition, Philadelphia and London, 1918.

[27] Ranson, S. W., *J. Comp. Neurol.*, 1912, xxii, 487.

[28] Rio Hortega, P., *Trab. lab. invest. biol. Univ. Madrid*, 1916, xiv, 19.

[29] Rubaschkin, W., *Arch. mikr. Anat.*, 1904, lxxiv, 575.

[30] Lee, A. B., Microtomist's vade-mecum, Philadelphia, 8th edition, 1921.

produced by the pressure necessary for the vascular injection of formalin (Mice 1 to 4) or by mechanical trauma. Of the nineteen guinea pigs examined, only three showed small hemorrhages into the brain substance. These were found in the medulla of Guinea Pig 1 and in the cervical region of the spinal cords of Guinea Pigs 15 and 16. In addition, a few hemorrhages were noted in the meningeal blood vessels but it was not possible to establish their existence *intra vitam*. All three rabbits were negative in this respect.

Edema with diffuse infiltration of fluid into the tissues about the blood vessels as illustrated, for example, in Warthin's Figs. 5 and 7 was not observed in any of the poisoned animals; but simple dilation of the perivascular and perineuronal spaces, with the contours of the latter unusually smooth and even, was seen in several cases. This dilation, however, was of variable degree and was noted as well in some of the normal animals and in a few of the specificity controls. We therefore regarded it as dependent upon factors difficult to delimit but including the condition of the animal at the time of fixation and the technique used.

Meningitis.—Meningitis with the margination of polymorphonuclear leucocytes and lymphocytes to the walls of the blood vessels, with slight thrombosis and infiltration of a few of these cells into the pia, was observed in five guinea pigs (Guinea Pigs 2, 5 to 7, 19) out of nineteen. In four of these (Guinea Pigs 2, 5 to 7) clumps of microorganisms having the morphology of diplococci (about 1.7 by 1.4 microns), not acid-fast but resisting decolorization after Gram, were found intercellularly. They were not seen in the controls, which exhibited no evidence of a similar type of meningitis, neither were they found in either mice or rabbits. From these observations it seemed improbable that the thrombosis and the meningitis reported under this heading were to be considered as resultant from *botulinus* poisoning.

Perivascular and Focal Infiltrations.—Only five of the mice (Mice 1 to 5) poisoned with the toxin exhibited perivascular infiltrations of mononuclear elements as described in the literature under the heading of botulism. Since, however, these lesions were found to be absent sometimes in mice equally affected by the poison (Mice 6 to 9) and were seen in several apparently normal control mice (Mice 13, 19, 31, 38, and 82), it is unlikely that they were originally

caused by the action of the toxin, although they may be more prone to occur in animals whose vitality is reduced by poisoning. They were not found in similarly poisoned guinea pigs, but were observed in apparently normal rabbits, in others suffering from botulism, and under a variety of experimental conditions. Certain protozoan-like parasites have been reported in association with lesions of this kind in rabbits[31-34] and we have found similar organisms in mice (including Mouse 4) which we have described elsewhere.[18]

Gas Bubbles.—Gas bubbles, like those mentioned by Warthin,[13] and having the same general distribution but devoid of bacteria, were observed in a small proportion of mice, guinea pigs, and rabbits suffering from botulism. They were absent in three guinea pigs (Nos. 17, 18, 19) specially treated with refiltered toxin to obviate any possibility of the transfer of bacteria. They were, moreover, noted in some of the controls, particularly in brains which were not removed immediately after death, so that in our material these bubbles cannot be considered to have developed in relation to the poisoning.

RESULTS.

The results of our observations indicate that, except for a slight degree of vascular engorgement, all the lesions which we have noted in the brains of mice, guinea pigs, and rabbits suffering from *botulinus* poisoning are readily susceptible of some explanation other than that they are produced by the direct action of the toxin upon the central nervous system. This absence of microscopic evidence of a central action of the toxin is not, of course, conclusive proof that the cells in the brain are not primarily involved; because it is quite conceivable that, owing to the delicacy of the nervous mechanism, the toxin might well act centrally without leaving any traces microscopically visible. But the observations do tend to show that, upon the histological side, there is no evidence inconsistent with the results of physiological experiments indicating that the site of action of the toxin is upon peripheral nerve terminals.

[31] Wright, J. H., and Craighead, E. M., *J. Exp. Med.*, 1922, xxxvi, 135.

[32] Doerr, R., and Zdansky, E., *Schweiz. med. Woch.*, 1923, liii, 349.

[33] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Acad.*, 1923, clxxvii, 985; *Compt. rend. Soc. biol.*, 1923, lxxxix, 1157.

[34] Goodpasture, E. W., *J. Infect. Dis.*, 1924, xxxiv, 428.

THE EPIDEMIOLOGY OF A RABBIT RESPIRATORY INFECTION.

I. Introduction.

By LESLIE T. WEBSTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In order to understand more clearly the phenomena which underlie the epidemic spread of bacterial disease in man, Flexner and his associates[1] and Topley with his collaborators[2] have studied experimentally a native animal enteric disease, mouse typhoid. In the course of these investigations an attempt has been made to reproduce mass reactions which arise in instances of human epidemics and to define the relative importance of such factors as microbic distribution, microbic virulence, and host susceptibility. Finally, on the basis of these studies, an hypothesis has been constructed to explain the spread of typhoid-like diseases in man and in animals.[3] This theory may be summarized as follows:

The bacteria of enteric infection are widespread and may be found in the intestinal contents of many hosts. Strains differ markedly in pathogenicity but the inherent virulence or pathogenicity of any given strain is relatively a fixed quantity, not affected by animal passage.

[1] Flexner, S., and Amoss, H. L., *Proc. Nat. Acad. Sc.*, 1921, vii, 319. Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9. Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15. Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45. Amoss, H. L., and Haselbauer, P.P., *J. Exp. Med.*, 1922, xxxvi, 107. Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71, 97; 1923, xxxvii, 21, 33, 231, 269, 781; xxxviii, 33, 45; 1924, xxxix, 129; *Am. J. Hyg.*, 1924, iv, 134. Pritchett, I. W., *J. Exp. Med.*, 1924, xxxix, 265.

[2] Topley, W. W. C., *Lancet*, 1919, ii, 1, 45, 91; *J. Hyg.*, 1920–21, xix, 350; 1921, xx, 103. Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227. Topley, W. W. C., *J. Hyg.*, 1922–23, xxi, 10, 20. Topley, W. W. C., and Wilson, G. S., *J. Hyg.*, 1922–23, xxi, 237, 243. Topley, W. W. C., *J. Hyg.*, 1922–23, xxi, 226. Topley, W. W. C., and Ayrton, J., *J. Hyg.*, 1923–24, xxii, 222, 234.

[3] Webster, L. T., *Am. J. Hyg.*, 1924, iv, 134.

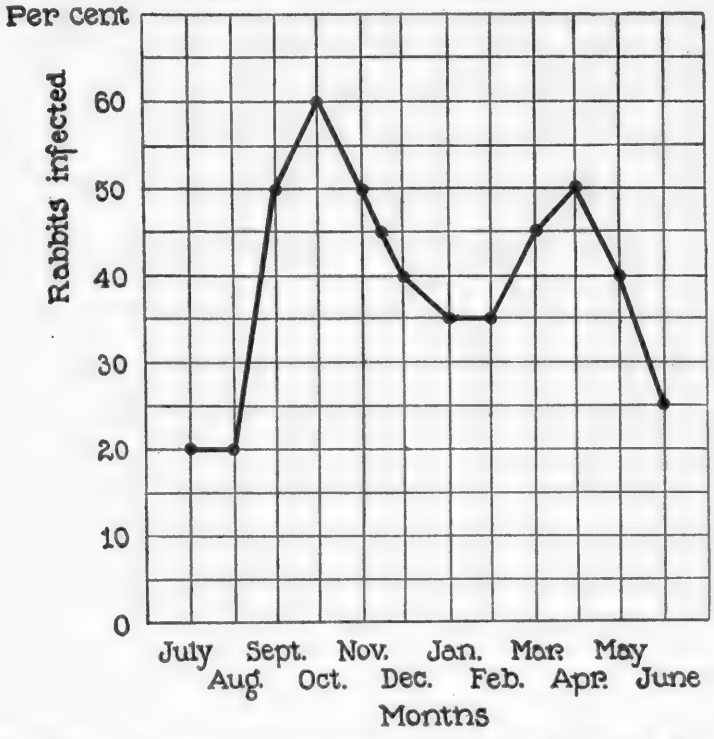
Adequate distribution of a strain with a certain degree of virulence is sufficient to induce an epidemic in a susceptible community. If distribution is gradual, the resulting mortality curve will be irregular; if distribution is sudden and general, the mortality curve will simulate a classical epidemic or skew frequency curve, the slope of which depends upon the individual susceptibilities of the population. In other words, virulence is relatively fixed; dosage and susceptibility are the two variables regulating the form of an epidemic curve. At the time of a pandemic, when microbic distribution is general and massive, dosage may also be considered constant and host susceptibility then becomes the one variable described by the epidemic curve.[3]

Experiments relating to the study of mouse typhoid have been simple; the causative microbes are quite well known and classified; they grow readily on plain media; they are antigenically active. The mouse host is quickly and easily obtainable in large numbers; breeding and living conditions, ancestry, age, and weight are easily controllable. Hence the study of mouse typhoid has served as valuable and illuminating training for the investigation of a more difficult problem dealing with the epidemic infections of the respiratory tract.

Just as the work on enteric epidemics followed upon an outbreak of mouse typhoid at The Rockefeller Institute, so the study of respiratory epidemics has been stimulated by the presence of respiratory infections throughout the rabbit stock at the Institute. These affections were originally and indeed are continually brought in from the outside. Rabbits are purchased from dealers located in New York, Pennsylvania, New Jersey, Ohio, etc., who assemble such animals from various amateur and professional breeders in their localities. The past history of each rabbit is therefore relatively unknown; its contact with infected rabbits or carriers cannot be detemined; indeed, its general health is a matter of question until a period of quarantine has elapsed. It is not surprising, therefore, that respiratory infections are common among laboratory rabbits.

These infections are difficult to classify. By far the most common of them is the mild respiratory affection, "snuffles"—a clinical term which is used to indicate a serous, mucoid, or purulent discharge from the nose. Breathing usually becomes audible, sneezing is frequent, and forepaws are damp and matted from wiping the discharge,

but aside from this, the animal appears normal. This affection is present throughout the year among the laboratory rabbits, but varies in frequency from month to month. In summer not more than 20 per cent of the entire stock show clinical signs. In September and October the percentage rises rapidly to 50 or 60, after which it drops gradually, registers another rise in March and April, and reaches the low level during July and August (Text-fig. 1).



TEXT-FIG. 1. Estimate of relative frequency of rabbit snuffles at The Rockefeller Institute.

There occur also during the year, among the stock and experimental animals, a few cases of chronic abscess and fatalities from pleuropneumonia, otitis media, meningitis, and septicemia. This group is small but its definite relation to the more prevalent affection, snuffles, becomes apparent as our studies progress.

The respiratory conditions in the rabbit, then, are quite similar to those occurring in man and give rise to the same problems. What is the relation between these various types of infection and what are the etiological agents concerned? What, also, are the microbic and host conditions which determine mild or severe infection, and sporadic, endemic, or epidemic prevalences? What factors are responsible for the evolution and devolution of the outbreaks and how do they operate?

The literature on this respiratory disease of rabbits is an index of the present confused state of knowledge.

As early as 1872, Davaine,[4] and in 1878, Koch,[5] described a spontaneous bacterial septicemia of rabbits. In 1881 Gaffky[6] gave a fuller description of the disease which he attributed to a non-motile coccus present in the blood, lungs, and pleural exudate.

In 1886, Hueppe[7] identified a single microorganism, the bacillus of hemorrhagic septicemia, as the bacterial incitant of swine-plague, chicken-cholera, and rabbit septicemia. Lignières (1901)[8] gave to this group of bacilli the present designation of Pasteurella and added to the known members those of the bovine, canine, and equine species.

The bacillus of rabbit septicemia (*Bacterium lepisepticum*) was minutely described by Smith in 1886,[9] and from this time on, many authors have considered this organism to be the cause of the rabbit epidemic respiratory disease characterized by nasal discharge, rapid collapse, and death with nasal passages filled with pus, pleuropneumonia, septicemia, and frequently pericarditis.

Recently, however, chronic snuffles has been differentiated etiologically from epidemic pleuropneumonia and septicemia. Ferry and Hoskins (1919)[10] say: "It seems to be evident that the ordinary form of snuffles . . . . is caused by *B. bronchisepticus*; while the more acute form and the most fatal, in the majority of instances is due to *Bacterium lepisepticum*. This statement is not based upon absolute findings,—but is the opinion of the authors." McCartney and

[4] Davaine, C., *Bull. Acad. Méd.*, 1872, i, series 2, 907; 1881, x, series 2, 249.

[5] Kock, R., *Untersuchungen über die Aetiologie der Wundinfectionskrankheiten*, Leipsiz, 1878, 59.

[6] Gaffky, G., *Mitt. k. Gsndhtsamite*, 1881, i, 80.

[7] Hueppe, F., *Berl. klin. Woch.*, 1886, xviii, 753, 776.

[8] Lignières, J., *Ann. Inst. Pasteur*, 1901, xv, 734; *Bull. Inst. Pasteur*, 1907, v, 249.

[9] Smith, T., *J. Comp. Med. and Surg.*, 1887, viii, 24; *J. Med. Research*, 1913–14, xxix, 291.

[10] Ferry, N. S., and Hoskins, H.P., *J. Lab. and Clin. Med.*, 1919–20, v, 311.

Olitsky consider that *Bacterium lepisepticum* cannot be considered as the essential incitant of experimental snuffles in ordinary laboratory stock rabbits because of preexisting nasal sinusitis in these animals.[11]

It is apparent, then, that the relation between snuffles and pleuropneumonia and septicemia is obscure and that the cause of chronic snuffles is still to be determined.

In subjecting the respiratory infections of rabbits to renewed study we have sought to use the experience gained in the experimental investigation of mouse typhoid. An essential feature of the study has been the breeding of rabbits at The Rockefeller Institute, where the history of the parent and offspring is known and the living conditions can be kept constant.

The nasal flora of all the rabbits in this breeding room is determined at frequent intervals and any animal which shows an abnormal flora, and any one developing suspicious respiratory symptoms, is at once discarded.

In addition, we are determining over long periods of time the nasal flora of old and young rabbits, both normal and infected, as they are employed for various purposes, with reference in particular to the features of parasitism and virulence of the pathogenic strains of *Bacterium lepisepticum* isolated. Moreover, we are paying particular attention to the clinical, pathological, and bacteriological manifestations of snuffles and to the circumstances under which the so called spontaneous affection arises. Finally, we are endeavoring to conduct this investigation into the epidemiology of this respiratory infection under circumstances as strictly controlled as in the corresponding study of mouse typhoid.

[11] McCartney, J. E., and Olitsky, P. K., *J. Exp. Med.*, 1923, xxxviii, 591.

THE EPIDEMIOLOGY OF A RABBIT RESPIRATORY INFECTION.

II. Clinical, Pathological, and Bacteriological Study of Snuffles.

By LESLIE T. WEBSTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Although there has been no acute epidemic respiratory infection among the rabbits at The Rockefeller Institute during the past few years, snuffles has prevailed widely among the laboratory stock.[1]

Clinical Findings.

Snuffles in rabbits as it occurs at this institution presents the following physical signs: coughing and sneezing, a nasal discharge, and matted forepaws.

Coughing and sneezing are irregular in duration and amount. The nasal discharge may be serous, mucoid, or purulent. When the discharge is serous, diagnosis is difficult, for a rabbit's nose is naturally moist and is often dampened by green food. A serous discharge, if accompanied by wet forepaws, may be considered a positive sign; if the paws are dry, further examination is necessary. A mucoid or purulent discharge suffices for diagnosis, for in these cases the forepaws are almost always wet or matted. Any moistening of the forepaws indicates a continuous discharge and is therefore very important in cases where diagnosis is uncertain. The more painstakingly these physical signs are looked for the greater is the percentage of positive diagnoses. Ordinarily no temperature changes have been observed; blood counts are not altered; nutrition and general condition seem to be normal.

The signs of snuffles may be transitory or intermittent, in which cases the serous discharge ceases and the purulent discharge dries up; or they may be progressive, when the serous discharge becomes purulent and endures sometimes over a period of years. This

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837.

Intermittent snuffles.								
Rabbit 8.		Presence of *Baci. lepisepticum.*	Rabbit 51.		Presence of *Baci. lepisepticum.*	Rabbit 82.		
Date.	Condition.	Nasal flora.	Date.	Condition.	Nasal flora.	Date.	Condition.	
1922			*1922*			*1923*		
Jan. 30	Well.	0	Mar. 3	Well.	+	Sept. 25	Well.	
Mar. 24	"	+	" 6	"	+	" 28	Serous discharg	
Apr. 12	"	+	" 9	"	+			
" 17	Snuffles +	+	" 13	"	+	Oct. 2	" "	
" 19	" +	+	" 20	"	+		One paw ma	
May 10	Well.	+	" 24	"	+			
" 19	"	+	Apr. 12	"	+	" 11	Serous discharg	
June 6	Snuffles +	+	" 17	Snuffles +	+			
" 21	Well.	+	May 10	Well.	+	" 16	" "	
July 14	"	+	Nov. 1	"	+			
Sept. 22	"	+	Dec. 4	"	+	" 18	Mucous "	
" 26	"	+	*1923*					
Nov. 1	Snuffles +	+	Jan. 9	Snuffles +	+	" 19	Snuffles ++	
Dec. 4	" ++	+	" 15	Well.	+	" 30	" ++	
1923						Nov. 19	" ++	
Jan. 9	" ++	+				" 20	Killed. Pus i	
" 15	" ++	+					nasal passag	

Snuffles + indicates slight snuffles, snuffles ++, severe snuffles. On each date i
Bacterium lepisepticum. The third column under each rabbit heading indicates the

Chronic snuffles.

Rabbit 75.		Presence of *Bact. lepisepticum*. Nasal flora.	Rabbit 18.		Presence of *Bact. lepisepticum*. Nasal flora.	Rabbit 48.		Presence of *Bact. lepisepticum*. Nasal flora.
Date.	Condition.		Date.	Condition.		Date.	Condition.	
1923			*1922*			*1922*		
. 25	Well.	0	Jan. 30	Well.	?	Jan. 30	Well.	0
28	"	0	Mar. 24	"	+	Mar. 24	"	
. 2	Serous discharge, slight.	0	Apr. 12	Snuffles +	+	Apr. 12	Snuffles ++	+
			" 17	" ++	+	" 17	" ++	+
			" 19	" ++	+	" 19	" ++	+
11	Same.	+	May 10	" ++	+	May 10	" ++	+
30	"	+	" 19	" ++	+	" 19	" ++	+
v. 2	Mucoid discharge.	+	June 6	" ++	+	June 6	" ++	+
			" 21	" ++	+	" 21	" ++	+
19	Snuffles ++	+	July 14	" ++	+	July 14	" ++	+
			Sept. 22	" ++	+	Sept. 22	" ++	+
			" 24	" ++	+	" 24	" ++	+
			Nov. 1	" ++	+	Nov. 1	" ++	+
			" 15	Died. Otitis media. Meningitis. *Bact. lepisepticum* in all cultures.	+	Dec. 4	" ++	+
						1923		
						Jan. 9	" ++	+
						" 15	" ++	+

condition of the animal was noted and a nasal swab made to test for the presence of
bsence of the organism in the nasal passages on the given date.

TABLE II.

Rabbits with Clinical Snuffles and Infected Nasal Passages.
Bacterium lepisepticum Present.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
1	Purulent ++	++	Liver, white spots.	Purulent ++	0	0	∞	0	0	∞	0	
3	" ++	++	Normal.	Seropurulent +	0	0	∞	0	0	∞	0	
4	" +	+	"	Purulent ++	0	0	∞	0	0	∞	0	
25	" +	+	"	Congested; mucous +	0	0	++	0	0	+		
28	" ++	++	"	Purulent ++	0	0	∞	0	0	∞		
56	Serous +	+	Liver, white spots +	Normal.	+	0	+	+	0	+	0	
59	" ?	+	Normal.	Congested.	0	0	++	0	0	++	0	
78	Seropurulent ++	++	"	"	0	0	∞	0	0	∞		
141	Purulent ++	++	Acute coccidiosis.	Mucopurulent +	0	0	∞	0	0	∞	0	0
144	Mucopurulent +	+	Normal.	Purulent ++	0	0	∞	0	0	∞	+	0
152	Purulent ++	++	Liver, white spots +	" ++	0	0	∞	+	0	∞		
153	" ++	++	Normal.	Mucopurulent +	0	0	∞	0	0	∞		
156	Mucous +	?	"	Congested; mucous.	+	0	∞	+	0	∞		
157	Purulent ++	++	"	Purulent ++	0	0	∞	0	0	∞	+	
158	" ++	++	"	" ++	0	0	∞	0	0	∞		
164	" ++	++	"	" ++	0	0	∞	0	0	∞		
165	" ++	++	"	" ++	0	0	∞	0	0	∞		
166	" ++	++	"	" ++	0	0	∞	0	0	∞		
168	" ++	++	Liver, white spots +	" ++	0	0	∞	0	0	∞		
169	" +	+	Normal.	" ++	0	0	∞	0	0	∞		
172	" ++	++	"	" ++	0	0	∞	0	0	∞		
173	" ++	++	"	" ++	0	0	∞	0	0	∞		

181	Serous +	+	Liver, white spots+	Purulent +	0	0	∞	0	0	∞		
184	Purulent ++	++	Lungs, consolidated areas.	" ++	0	0	∞	0	0	∞		
185	Serous +	+	Normal.	Congested.	0	0	∞	0	0	∞		
186	Purulent ++	++	"	Purulent ++	0	0	∞	0	0	∞	0	0
188	" ++	++	"	" ++	0	0	∞	0	0	∞	+	0
197	" ++	++	"	" ++	0	0	∞	0	0	∞	0	0
198	" ++	++	"	Mucous ++	0	0	∞	0	0	∞	0	0
199	" ++	++	"	Purulent ++	0	0	∞	0	0	∞	0	0
200	" ++	++	Liver, white spots.	" ++	0	0	∞	0	0	∞	0	0
202	" ++	++	Normal.	" ++	0	0	∞	0	0	∞	0	0
205	Serous +	++	"	" ++	0	0	∞	0	0	∞	+	0
206	Purulent ++	++	"	" ++	0	0	∞	0	0	∞	0	+
207	Serous +	+	"	Mucopurulent +	0	0	∞	0	0	∞	0	+
211	Purulent ++	+	"	Purulent ++	+	0	∞	+	0	∞	0	0
213	" ++	++	"	" ++	0	0	∞	0	0	∞	0	0
82	" ++	++	"	" ++	++	0	∞	++	0	+	0	0
75	" ++	++	"	" ++	0	0	∞	0	0	∞	0	0
91	" ++	++	"	" ++	++	0	∞	++	0	∞	0	0

C. indicates cocci; Br., *B. bronchisepticus;* L., *Bacterium lepisepticum.* +, in Columns 2–5, indicates moderate; ++, abundant; +, in Columns 6–13, indicates 1–10 colonies; ++, 10–100 colonies; ∞, over 100 colonies.

latter is the usual variety and is detected by the audible breathing, the sneezing, and the purulent discharge from the nose which mats the forepaws. Table I outlines the clinical histories of two rabbits with intermittent snuffles, two in which the disease was progressive, and two in which it became chronic.

Pathological and Bacteriological Findings.

In the attempt to relate these clinical findings with definite anatomical changes and specific bacteria, 100 adult rabbits with and without snuffles were autopsied and cultured. These rabbits had been generally lively and in good condition. A few of them had been in the animal house but 2 or 3 days; most of them, however, had been there for 1 month at least. The rabbits were killed by a sharp occipital blow and autopsied immediately. The condition of heart and lungs was noted, 0.5 cc. of heart's blood was inoculated into 5 cc. broth, pH 7.4, and a generous piece of lung was placed in another tube of broth. The abdominal organs were then examined. Finally, sterile instruments were used to open wide the nasal passages and antra. Sterile cotton swabs, one for each side, were rubbed over all the nasal mucous surfaces and then streaked upon large 14 cm. Petri dishes containing plain agar, pH 7.4, with 5 per cent defibrinated whole rabbit blood. The plates had been poured not more than 2 or 3 hours before use.

It is important to adhere to this technique.[2] Swabs are necessary for a thorough bacteriological examination of the nasal passages—a platinum loop will not do. Unless the plates are prepared as described and are used immediately, many varieties of *Bacterium lepisepticum* will not grow. Horse serum agar, plain agar, and dextrose agar cannot be used. Finally, the plates require in many instances a most painstaking examination. Many colonies of *Bacterium lepisepticum* are indistinguishable from *B. bronchisepticus* and repeated smears and hanging drops from many colonies are often required in order to obtain accurate information.

[2] The procedure for culturing the nasal passages is given in detail because the results obtained by this method reveal a difference in the nasal flora from that found by McCartney and Olitsky who employed suspensions of the entire mucous membrane for culture. They report "in the order of their frequency, *Staphylococcus albus*, *Bacillus bronchisepticus*, *Bacillus lepisepticus*, *Micrococcus catarrhalis*" (*J. Exp. Med.*, 1923, xxxviii, 596).

The pathological findings at autopsy, aside from hemorrhagic areas in the lungs and scarred or spotted livers, were usually limited to the nasal passages. Here the anatomical changes usually paralleled the clinical findings. The lumina were clean or contained mucus or thick, firm pus in varying amount. The mucosa was transparent, or injected, or thickened and rubbery. The turbinates and nasal septa were delicate or thickened, eroded, or atrophic.

The bacteriological findings may best be described by correlating them with the clinical and pathological conditions. This has been done by means of a series of tables in which the protocol of each animal is summarized. Nasal discharge and condition of paws are noted; condition of viscera and appearance of nasal passages are recorded; and the results of the bacteriological examination are given, with special emphasis on the presence or absence of *Bacterium lepisepticum* and *Bacillus bronchisepticus*.

In Table II are grouped the data on forty rabbits with clinical snuffles in various stages of severity. Occasionally, a serous nasal discharge was recorded, but in most of the cases it was decidedly purulent in character. Considerable matting of the forepaws was noted in all but one case. At autopsy, seven rabbits showed focal liver necroses; one showed consolidated areas in its lungs. The pathology of the nasal passages varied from a congestion of the mucous membrane, five cases, to lumina and antra filled with pus and turbinates eroded or atrophic, thirty-five cases. The bacteriology of this group was uniform; *Bacterium lepisepticum* was cultured as the predominating ogranism from both nasal passages in every case. Cocci were few in number and when present usually proved to be *Micrococcus catarrhalis* or *Staphylococcus albus*; *Bacillus bronchisepticus* was not seen. *Bacterium lepisepticum* was grown from the heart's blood of four rabbits and from the lungs of two.

Table III records eight similar cases of clinical snuffles, two of which at autopsy showed focal liver necroses. The nasal mucosa of four was congested and covered with mucoid pus; the other four cases showed advanced chronic inflammation with eroded turbinates and passages filled with pus. *Bacterium lepisepticum* and *Bacillus bronchisepticus* were cultured from both nares of these cases in about equal numbers. Cocci occurred as before.

TABLE III.

Rabbits with Clinical Snuffles and Infected Nasal Passages.
Bacterium lepisepticum and B. bronchisepticus Present.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
46	Purulent ++	++	Liver, white spots ++	Purulent ++	0	∞	∞	0	∞	∞		
49	" ++	++	" " " and scarring.	Congested.	0	∞	∞	0	++	++		
57	Serous +	+	Normal.	Congestion; mucous +	+	++	++	+	++	++	0	0
58	Seropurulent +	+	"	Congested; mucopurulent +	0	+	∞	0	+	∞		
155	Purulent +	+	"	Congested; mucous +	0	++	++	0	++	++		
215	" ++	++	"	Purulent ++	+	++	∞	+	++	∞	0	0
90	" ++	++	"	" +	++	++	++	++	++	++	0	0
358	" ++	++	"	" ++	+	++	++	++	++	++	0	0

In Table IV are grouped three important cases. The first two showed clinical snuffles and one of them, No. 139, showed definite lesions of the nasal passages. But although nasal cultures during life had repeatedly shown *Bacterium lepisepticum* and *Bacillus bronchisepticus*, the cultures at autopsy showed only *Bacillus bronchisepticus*. Old culture medium was used in these two cases and was probably responsible for this discrepancy. Rabbit No. 170 was a questionable case clinically with doubtful pathological lesions of the nasal passages. *Bacillus bronchisepticus* alone was cultured at autopsy. These three cases are included for the sake of completeness and to show how easily some fault in technique and clinical diagnosis may be misleading.

TABLE IV.

Rabbits with Clinical Snuffles and Infected Nasal Passages. B. bronchisepticus Present.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
139*	Purulent ++	++	Normal.	Purulent ++	0	++	0	0	++	0	0	0
145*	Serous +	+	"	Normal.	0	∞	0	0	∞	0	0	0
170†	" +	+	"	Congested; mucous?	0	∞	0	0	∞	0		

*Nasal cultures before death showed *B. bronchisepticus* and *Bacterium lepisepticum*.

†Diagnosis of snuffles in this case is somewhat uncertain.

Table V is also of especial interest. In it are described four cases of unilateral snuffles. Of these, three showed a unilateral nasal discharge, matting of the corresponding forepaw, and, at autopsy, a definite inflammatory condition of the affected nasal passage from which *Bacterium lepisepticum* was isolated in almost pure culture. The other nasal passage appeared normal in each case and showed no *Bacterium lepisepticum*. From one of these normal passages, however, *Bacillus bronchisepticus* was cultured. The fourth case presented a matted left paw, an inflamed condition of the left nasal passage, and colonies of *Bacterium lepisepticum* from both passages.

TABLE V.

Rabbits with Clinical Snuffles and Infected Nasal Passage on One Side Only. Bacterium lepisepticum Recovered from Affected Side.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
2	Left nares, purulent ++	Left ++	Intestinal adhesions.	Right side normal; left side purulent +	0	∞	0	0	0	∞	0	
27	Left nares, purulent +	" +	Normal.	Normal.	0	0	0	0	0	+	0	
195	Right nares, serous +	Right +	"	Right mucous +; turbinate thickened; left congested.	0	0	∞	++	0	0	0	0
228	Normal.	Left +	"	Right normal; left congested; turbinate thickened.	++	0	+	++	0	+	0	0

This group of tables, (II to V), then, summarizes a random series of 55 cases of snuffles from the nasal passages of which *Bacterium lepisepticum* was obtained as the predominating organism in all but the three cases in which error in diagnosis and technique seemed to account for the discrepancy. *Bacillus bronchisepticus* was cultured from these three cases and also from eight others of this series.

Table VI shows the findings in three rabbits with little or no evidence of clinical snuffles but which, at autopsy, presented nasal passages filled with pus. *Bacterium lepisepticum* was cultured from both nasal passages of all and from the blood of one and the lungs of two animals. These cases are included as tending to confirm the

TABLE VI.

Rabbits with No Clinical Snuffles but with Infected Nasal Passages. Bacterium lepisepticum Recovered.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
201	Serous?	Normal.	Normal.	Mucopurulent +	0	0	∞	0	0	∞	0	+
203	" ?	"	"	Purulent ++	0	0	∞	0	0	∞	+	+
204	" ?	"	"	" ++	0	0	∞	0	0	∞	0	0

statement of McCartney and Olitsky[8] that in stock rabbits a chronic inflammation of the nasal passages may be present with few or no clinical signs.

Table VII shows data on three rabbits, normal clinically and on gross autopsy inspection, from which both *Bacterium lepisepticum* and *Bacillus bronchisepticus* were cultured. Table VIII gives the findings in five similar normal animals from which *Bacterium lepisepticum* was cultured but no *Bacillus bronchisepticus*. Table IX records nineteen similar normal cases from which *Bacillus bronchisepticus* was cultured but no *Bacterium lepisepticum*. Table X describes fifteen normal ones from which neither *Bacterium lepisepticum* nor *Bacillus bronchisepticus* was recovered.

[8] McCartney, J. E., and Olitsky, P. K., *J. Exp. Med.*, 1923, xxxviii, 591.

This group of tables (VII to X) is representative of a series of forty-two rabbits whose upper respiratory tract appeared normal clinically and at gross autopsy inspection. In eight of these cases

TABLE VII.

Rabbits Clinically and Anatomically Normal.
Bacterium lepisepticum and B. bronchisepticus Recovered.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
48*	None.	Normal.	Normal.	Normal.	0	∞	0	0	0	∞		
221	"	"	"	"	+	∞	∞	+	∞	∞	0	0
223	"	"	Liver, coccidiosis ++	"	0	∞	∞	0	∞	∞	0	0

* Abscess on leg. *Bacterium lepisepticum* +.

TABLE VIII.

Rabbits Clinically and Anatomically Normal.
Bacterium lepisepticum Recovered.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
45	Serous ?	Normal.	Normal.	Slight congestion.	0	0	∞	0	0	+		
127	None.	"	"	Normal.	+	0	∞	+	0	∞	0	
194	"	"	∞	Congested.	0	0	∞	0	0	∞	0	0
196	"	"	"	"	++	0	++	++	0	++	0	0
226	"	"	"	Normal.	+	0	∞	+	0	∞	0	0

Bacterium lepisepticum was cultured. *Bacillus bronchisepticus* was found in twenty-two cases, and in nineteen cases neither organism was recovered.

TABLE IX.

Rabbits Clinically and Anatomatically Normal.

B. bronchisepticus Recovered.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
					Right nasal passage.			Left nasal passage.				
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	C.	Br.	L.	C.	Br.	L.	Blood.	Lung.
47	Serous ?	Normal.	Normal.	Normal.	+	∞	0	+	∞	0	0	
154	None.	"	"	"	+	++	0	+	++	0		
159	"	"	"	Congested.	+	∞	0	+	∞	0		
167	"	"	"	"	++	+	0	++	+	0		
171	Serous +	?	"	Normal.	+	++	0	+	++	0		
182	" ?	Normal.	"	Congested ?	0	++	0	0	++	0		
192	None.	"	"	Normal.	+	∞	0	+	∞	0	0	0
208	"	"	Liver, white spots.	"	+	∞	0	+	∞	0	0	0
209	"	"	" " " +	Congested.	+	∞	0	+	∞	0	0	0
210	"	"	Normal.	Normal.	+	∞	0	+	∞	0	0	0
212	"	"	"	Congested ?	+	∞	0	+	∞	0	0	0
214	Serous ?	"	"	Normal.	+	∞	0	+	∞	0	0	0
216	None.	"	"	"	++	∞	0	++	∞	0	0	0
217	"	"	Liver, white spots +	"	+	∞	0	+	∞	0	0	0
218	"	"	Normal.	"	+	∞	0	+	∞	0		
220	"	"	"	"	+	∞	0	+	∞	0		
222*	"	"	Liver, coccidiosis ++	"	+	∞	0	+	∞	0		
224	"	"	" " +	"	0	∞	0	0	∞	0	0	0
225	"	"	Normal.	"	+	∞	0	+	∞	0	0	0

*Abscess on back. *Bacterium lepisepticum* +.

TABLE X.

Rabbits Clinically and Anatomically Normal.
No Bacterium lepisepticum and No B. bronchisepticus Recovered.

Rabbit No.	Clinical condition.		Autopsy findings.		Bacteriological findings.							
	Nasal discharge.	Paws.	Viscera.	Nasal passages.	Right nasal passage.			Left nasal passage.			Blood.	Lung.
					C.	Br.	L.	C.	Br.	L.		
26	Serous ?	Normal.	Liver, white spots ++	Normal.	+	0	0	+	0	0	0	
96	" ?	"	Normal.	Congested ?	+	0	0	+	0	0	0	
97	" ?	?	Liver, white spots +	" ?	+	0	0	+	0	0	0	
98	Normal.	Normal.	Normal.	" ?	+	0	0	+	0	0	0	
142	None.	"	"	Normal.	+	0	0	+	0	0	0	0
147	"	"	"	Congested.	+	0	0	+	0	0	0	0
148	"	"	"	Normal.	+	0	0	+	0	0	0	0
151	"	"	"	Congested.	+	0	0	+	0	0	0	0
160	Mucous ?	"	"	"	++	0	0	++	0	0		
174	None.	"	"	"	+	0	0	+	0	0	0	0
193	"	"	"	"	+	0	0	+	0	0	0	0
219	"	"	Liver, white spots.	Normal.	++	0	0	+	0	0	0	0
92	"	"	Normal.	Congested.	++	0	0	++	0	0	0	0
227	"	"	"	Normal.	+	0	0	+	0	0	0	0
229	"	"	"	Congested.	∞	0	0	∞	0	0	0	0

SUMMARY.

100 adult rabbits taken at random from The Rockefeller Institute animal house were examined clinically, anatomically, and bacteriologically for evidence of respiratory infection. 58 of these rabbits proved to have snuffles; 42 were free of the disease.

Bacterium lepisepticum was the predominating organism in the nasal flora of 55 of the 58 rabbits showing snuffles. This organism was also cultured from the nasal passages of eight normal rabbits.

Bacillus bronchisepticus was associated with *Bacterium lepisepticum* in the nasal flora of eight rabbits with snuffles. It was also cultured from the nares of twenty-two normal rabbits. Fifteen normal rabbits showed neither *Bacterium lepisepticum* nor *Bacillus bronchisepticus*.

THE EPIDEMIOLOGY OF A RABBIT RESPIRATORY INFECTION.

III. Nasal Flora of Laboratory Rabbits.

By LESLIE T. WEBSTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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As a part of the investigation into the etiology of rabbit snuffles, a study has been made on the nasal flora of rabbits during the past 2 years. In this paper there will be described three typical series of observations to illustrate the method employed and the data regularly obtained throughout the work. Finally, the findings from this and the preceding paper[1] will be employed to arrive at a tentative conclusion as to the cause of rabbit snuffles at The Rockefeller Institute.

Method.

The nasal flora of the rabbit has been studied by culturing both nares of the same individuals at frequent intervals over long periods of time. The different types of aerobic bacteria and the relative frequency of each have been determined. The exact technique may be described as follows:

The rabbit is held by an attendant. One of the operator's hands holds the rabbit's head, and his other hand passes a sterile cotton swab up into the nasal passage, 1 to 3 cc., depending upon the size of the rabbit. The swab is revolved slowly so as to cover all available portions of the nares. It is then withdrawn and a fresh swab is used for the opposite side.

The swabs are streaked thoroughly over 14 cc. Petri dishes containing freshly prepared blood agar (pH 7.4 + 5 per cent defibrinated rabbit blood). These plates are incubated 24 hours and then examined. Colonies are identified by smear, hanging drop, and culture methods, and the relative numbers of each colony are estimated.

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[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 843.

At least three tests over an interval of 1 week are considered necessary to give accurate information concerning the nasal flora of a rabbit at a given time. When this procedure has been carried out, the findings will invariably check with subsequent autopsy cultures and may be used as a safe index to interpret the present and predict the future behavior of the rabbit's respiratory tract.

Three series of experiments are reported in this paper. The first series deals with the flora of purchased rabbits immediately upon their arrival at the Institute; the second series relates to the flora of stock Institute rabbits which have been on hand at least 1 month; and the third series to the flora of rabbits which are being used for experimental purposes.

Bacteria Present in the Nasal Passages.

The aerobic bacteria recovered from the nasal passages of rabbits examined at The Rockefeller Institute may be enumerated as follows: *Bacterium lepisepticum*, *Bacillus bronchisepticus*, Gram-negative cocci of five types, *Staphylococcus aureus* and *Staphylococcus albus*, hemolytic and non-hemolytic streptococci, and various organisms of the intestinal group, such as *Bacillus coli*, *Bacillus alkaligenes*, and *Bacillus aerogenes*.

Descriptions of the cultural and serological characters of *Bacterium lepisepticum* and *Bacillus bronchisepticus* will be published later.

The Gram-negative cocci of Type I answer all the requirements of the *Micrococcus catarrhalis* group. The colonies are small, dry, and convex. The cocci are irregular in form with many biscuit and diplo groups. They are non-motile. In broth, the growth is granular. Dextrose, lactose, saccharose, maltose, mannite, and salicin are not fermented. Milk is not changed, indole is not formed, gelatin it not liquefied.

Type II Gram-negative cocci form flat, wrinkled, irregular colonies. They are non-motile. The growth in broth is diffuse. Dextrose, lactose, saccharose, maltose, and mannite are fermented; salicin is not changed. Milk is turned slightly acid and tends to be peptonized. Indole is not formed; gelatin is not liquefied.

Type III Gram-negative cocci are quite similar to Type II except that salicin is fermented and mannite is not fermented. Milk is turned slightly acid.

Type IV differs only in its sugar reactions. Dextrose, saccharose, maltose, and salicin are fermented while lactose and mannite remain unchanged.

Type V resembles many types of staphylococci in that all sugars (dextrose, lactose, saccharose, maltose, mannite, and salicin) are fermented.[2] But they are

[2] Gordon, J. E., *J. Infect. Dis.*, 1921, xxix, 462.

Gram-negative. Growth in bouillon is diffuse. They are non-motile. Milk is turned acid and coagulated. Indole is not formed, and gelatin is not liquefied.

The staphylococci usually ferment all sugars. *Staphylococcus aureus* and *Staphylococcus albus* are found. *Micrococcus tetragenus* and Sarcinæ are occasionally noted. Hemolytic and non-hemolytic streptococci are present.

B. coli communis and *communior* have been isolated and *B. alkaligenes* and *B. aerogenes*. Gram-negative rods resembling *B. morgani* have been quite frequent and occasionally a member of the paratyphoid-enteritidis group has been found. All of these intestinal organisms are probably only present at the external nares, for they have not been found at autopsy when the deeper nasal passages are cultured.

Series 1. Purchased Rabbits.—The animals, after being received, are kept in special rooms until required for experimental purposes. The nasal flora was determined by a series of three or four cultures begun on the day of arrival and terminated within a week.

Group A consists of fifteen rabbits, weighing 1,000 to 1,500 gm., obtained from a dealer. They showed no clinical evidence of snuffles during the period of examination.

Table I summarizes the nasal flora of these rabbits. The *Micrococcus catarrhalis* type was present in large numbers in 66 per cent of the cases. *Bacterium lepisepticum* was the predominating organism in 46 per cent. *Bacillus bronchisepticus*, Gram-negative cocci of Type II, and unidentified intestinal bacilli in the lactose-fermenting group were present in fair numbers in 40 per cent of the cases. Type III Gram-negative cocci were present in 26 per cent. Occasional types were *Staphylococcus albus*, *Bacillus aerogenes*, *Bacillus coli*, *Bacillus alkaligenes*, *Bacillus morgani*, and the *Bacillus levans*.

Group B consists of 77 rabbits, weighing 500 to 1,000 gm., brought from The Rockefeller Institute farm in New Jersey. They showed no signs of snuffles during the period of observation. Table II summarizes the nasal flora of these rabbits.

The *Micrococcus catarrhalis* type was present in large numbers in 79.3 per cent of the cases; Type II Gram-negative cocci were found in large numbers in 44.2 per cent; *Bacterium lepisepticum* and *Bacillus bronchisepticus* in large numbers in about 25 per cent; Type III Gram-negative cocci and unidentified lactose-fermenting organisms of the intestinal group in about 14 per cent. Other types noted very occasionally were *Staphylococcus albus*, *Bacillus coli*, non-hemolytic

TABLE I.

Summary of Nasal Flora of Unknown "Outside" Rabbits.

Rabbit No.	*Baci. leptiseptieum.*	*B. bronchisepticus.*	Gram-negative cocci.					Staphylococci.		Streptococci.		Intestinal group.							Miscellaneous.
												Lactose fermenters.			Non-lactose fermenters.				
			Type 1. *Coiarrhalis.*	Type 2.	Type 3.	Type 4.	Type 5.	*Aureus.*	*Albus.*	Non-hemolytic.	Hemolytic.	*B. aerogenes.*	*B. coli.*	Unidentified.	Paratyphoid-enteritidis.	*B. alkaligenes.*	*B. morgani* type.	Unidentified.	
341		∞										++							
345		∞			++				+										
352		∞		+													+		
353	∞		+	++										+					
354			++	∞	++									+					
356	∞		++	+												+			
357			∞	++										+					
362		++	++		∞														
363	∞	+																	
370	∞	++	+	+										+					
375	∞		++																++
379													∞						
383	∞		+																
384	∞		++											++					
395			++		∞									+					
Total.																			
15	7	6	10	6	4				1			1	1	6		1	1		1
Per cent.																			
100	46	40	66	40	26				6			6	6	40		6	6		6

+ indicates 1–10 colonies; ++, 10-100 colonies; ∞, more than 100 colonies.

streptococci, *Bacillus aerogenes*, Gram-negative cocci of Type IV, *Staphylococcus aureus*, *Bacillus alkaligenes*, and unidentified lactose-fermenting organisms of the intestinal group.

The flora of these two outside groups are quite similar. The *Micrococcus catarrhalis* type is the most frequent and is found in about 70 per cent of the cases. Other Gram-negative cocci, *Bacterium lepisepticum*, and *Bacillus bronchisepticus* are present in about 35 per cent, and still other cocci and intestinal forms in decreasing frequency.

Series 2. Stock Rabbits.—Normal stock rabbits are those which have been kept at the Institute for at least a month. The first thirty-three animals in this series have not been used for experimental purposes and during a long period of observation have shown no clinical signs of snuffles. They have been kept in a breeding room free from snuffles and *Bacterium lepisepticum* carriers. Any animal in this group developing clinical signs of snuffles or *Bacterium lepisepticum* in the nasal passages was immediately killed and autopsied. Table III summarizes the average findings of many cultures on these rabbits.

Table III shows that all the thirty-six rabbits in this series showed the *Micrococcus catarrhalis* group in large numbers, 39 per cent showed *Bacillus bronchisepticus*, and 15 per cent *Bacterium lepisepticum*. The remaining bacteria were quite similar to those of other groups described. The low percentage of *Bacterium lepisepticum* in this series and the entire absence of clinical snuffles are accounted for by the fact that all carriers were immediately removed from the room.

The second group is composed of twenty-one adult rabbits which, a year ago, had received three small intravenous doses of a killed culture of *Bacterium lepisepticum*. This injection was quite ineffectual and the animals have since been kept under observation as normal control stock. Table IV summarizes the flora of these rabbits.

The summary of the nasal findings of the twenty-one rabbits in Group II shows that the *Micrococcus catarrhalis* group was present in 95 per cent of the cases, and *Bacterium lepisepticum* and *Bacillus bronchisepticus* in 70 per cent and 50 per cent, respectively. The remainder of the flora showed no unusual variation.

TABLE II.

Summary of Nasal Flora of Princeton Farm "Outside" Rabbits.

Rabbit No.	*Bact. lepisepticum.*	*B. bronchisepticus.*	Gram-negative cocci.					Staphylococci.		Streptococci.		Intestinal group.							Miscellaneous.
												Lactose fermenters.			Non-lactose fermenters.				
			Type 1. *Catarrhalis.*	Type 2.	Type 3.	Type 4.	Type 5.	*Aureus.*	*Albus.*	Non-hemolytic.	Hemolytic.	*B. aerogenes.*	*B. coli.*	Unidentified.	Paratyphoids-enteritidis.	*B. alkaligenes.*	*B. morgani* type.	Unidentified.	
186	∞																		
187			∞																
188	∞	+																	
189		+	∞																
190			∞						+										
191			∞											+					
207	∞		+																
208		∞	++																
209			+																
210	∞								++										
211	∞																		
212	∞		++																
213	∞																		
214		++	+																
215	++	∞																	
216		∞		++															
217		++																	
308		∞		+										++					
318		∞			++														

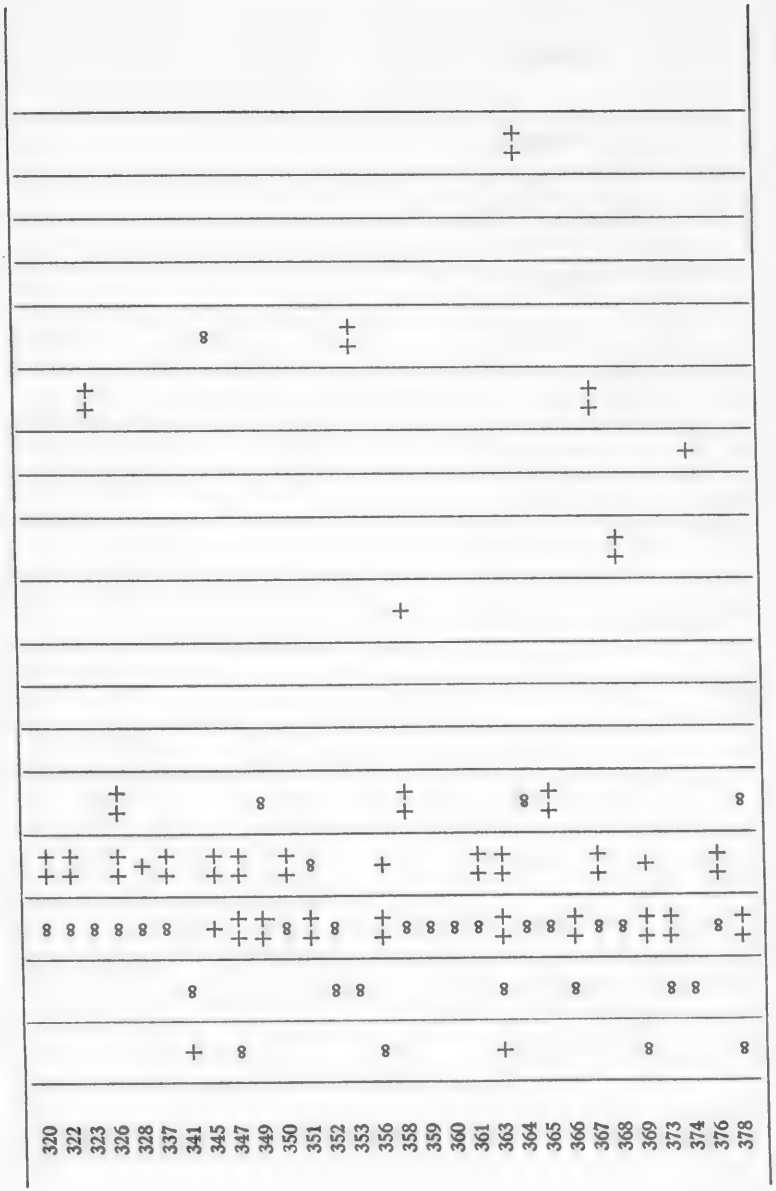
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TABLE II—*Concluded.*

Rabbit No.	*Bact. lepisepticum.*	*B. bronchisepticus.*	Gram-negative cocci.					Staphylococci.		Streptococci.		Intestinal group.							Miscellaneous.
												Lactose fermenters.			Non-lactose fermenters.				
			Type 1. *Catarrhalis*	Type 2.	Type 3.	Type 4.	Type 5.	*Aureus.*	*Albus.*	Non-hemolytic.	Hemolytic.	*B. aerogenes.*	*B. coli.*	Unidentified.	Paratyphoid-enteritidis.	*B. alkaligenes.*	*B. morgani* type.	Unidentified.	
379		∞							∞										
380	∞		++						+										
381		∞																	
382		++	++	∞															
383	∞			+										+					
385			∞		++								+						
386			∞	+										+					
387			++																
388			∞		∞											+			
389				++															*B. levans.*
391	∞		++	+	++									+					
392	∞		∞																+
394	∞		∞	∞															
395	++	∞	++																
396			∞	+						++									
397			∞		++		∞												
398			∞	++					+					+					
399			∞	++										+					
400			∞	++				+											
471			∞	+															

| | | |

TABLE III.

Summary of Nasal Flora of Normal Stock Rabbits.

Group 1.

Rabbit No.	*Baci. lepisepticum.*	*B. bronchisepticus.*	Gram-negative cocci.					Staphylococci.		Strepto-cocci.		Intestinal group.								Miscellaneous.
												Lactose fermenters.			Non-lactose fermenters.					
			Type 1. *Catarrhalis.*	Type 2.	Type 3.	Type 4.	Type 5.	*Aureus.*	*Albus.*	Non-hemolitic.	Hemolytic.	*B. aerogenes.*	*B. coli.*	Unidentified.	Paratyphoid-enteritidis.	*B. alkaligenes.*	*B. morgani* type.	Unidentified.		
50		+	±										+							
51		++	+																	
52		++	±	++																
53		+	±										+							
54		+	±														+			
55			±						+											
56*	±		++									+								
57*	++	++	±						+											
58*	±	+	++										+							
59*	++		++						+											
60		++	++										+							
61		+	±	++													+			Sarcinæ.
62			++		+															*Micrococcus tetra genus.*
63		++	++											+						
64			±																	
65*	±	+	++																	
66		++	++																	
67			++						+					+						*Pyocyaneus.*

68			++											+				
69			∞															
70			∞						+									
71			∞	+								+		+				
73*			++															
101			++						+					+				
102			∞		++				++									Sarcinæ.
103			∞															
104			∞	++														
105			∞	++														
106			++		++				+			+						
107			++															
108			∞	++	+				+			+						Sarcinæ.
110			∞															
111		++	++	+														
114			∞	+														
115			++	++														
117			∞	++	++													
Total.																		
36	5	13	36	10	5				7			4	4	4			2	5
Per cent.																		
100	15	39	100	30	15				21			12	12	12			6	15

* Killed. Autopsy cultures checked with those taken during life.

TABLE IV.

Summary of Nasal Flora of Normal Stock Rabbits.

Group 2.

Rabbit No.	*Bact. lepisepticum.*	*B. bronchisepticus.*	Gram-negative group.					Staphylococci.		Strepto-cocci		Intestinal group.							Miscellaneous.
												Lactose fermenters.			Non-lactose fermenters.				
			Type 1. *Coliorrhalis.*	Type 2.	Type 3.	Type 4.	Type 5.	*Aureus.*	*Albus.*	Non-hemolytic.	Hemolytic.	*B. aerogenes.*	*B. coli.*	Unidentified.	Paratyphoid-enteritidus.	*B. alkaligenes.*	*B. morgani* type.	Unidentified.	
74		++	∞	+										+					
75*	∞		++						+H.†										
76	++	+	++						++H.									+	
77		∞	+																
78*	∞	+	+																
79	∞		++																
80*	∞		++	+															
81			++	+			++					+							
82*	∞		++																
83		++	++																Gram-positive bacillus. Chromogenic.
84			++																
85*	∞		++																
86	∞		++	+	++									+					
87		+	++		+														
88*	∞		+															+	

90	++	++	++												+				
91*	∞		+																
92*	++	+	++																
93	++		++																
94	++	++						+											
95		∞	++																
Total.																			
21	14	10	20	4	2		1	1	2			1		2	1			2	1
Per cent.																			
100	70	50	95	20	10		5	5	10			5		10	5			10	5

* Developed snuffles during the period of observation. At autopsy cultures from the nasal passages checked with those taken during life.

† H. indicates hemolytic.

In brief, then, normal stock rabbits kept at the Institute for varying periods of time showed nothing unusual in the flora of their nasal passages except for a great frequency of *Bacterium lepisepticum.*

Series 3. Experimental Rabbits.—The data obtained from Series 1 and 2 describe the types and relative frequency of bacteria found in the nasal passages of normal rabbits. This experimental series represents a group of forty-two rabbits inoculated with tumor or syphilis by Drs. Brown, Pearce, and Van Allen and studied bacteriologically by us.

The bacteriological study was carried out in the routine way. As the variety and number of cocci and intestinal bacteria so nearly resembled those in the other series, the tables of this series group the different bacteria under three heads: (1) cocci, (2) colon group, and (3) *Bacillus bronchisepticus* and *Bacterium lepisepticum,* and present the general result of each observation rather than a detailed average observation as before.

These tables (V–IX) show variations in the flora of these rabbits over a period of 3 months for comparison with the final cultures taken at autopsy. In every case autopsy cultures checked the *Bacterium lepisepticum* and *Bacillus bronchisepticus* findings during life.

In Table V, serial cultures on eight normal rabbits are recorded whose nasal passages showed at one time or another both *Bacterium lepisepticum* and *Bacillus bronchisepticus.* According to this series, either the one or the other may disappear and later may recur.

Table VI shows four normal rabbits which remained free of *Bacterium lepisepticum* and *Bacillus bronchisepticus* during the entire period of observation.

Table VII tabulates the flora of nine normal rabbits which showed *Bacillus bronchisepticus* but no *Bacterium lepisepticum,* and Table VIII presents the records of ten normal rabbits showing *Bacterium lepisepticum* but no *Bacillus bronchisepticus* during the 3 months study.

Table IX groups the rabbits in this series with clinical snuffles, eleven in all, and shows that in each case *Bacterium lepisepticum* is the predominating organism. *Bacillus bronchisepticus* may also be present at one time or another, but when serial quantitative cultures are taken in this manner its presence appears to be quite unimportant.

TABLE V.

Experimental Rabbits. No Snuffles. Bacterium lepisepticum and B. bronchisepticum Present.

| Rabbit No. | Date of examination. | | |

TABLE VI.

Experimental Rabbits. No Snuffles. No Bacterium lepisepticum or B. bronchisepticus Present.

Rabbit No.	Date of examination.																															
	1923, Sept. 3rd wk.				1923, Sept. 4th wk.				1923, Oct. 3rd wk.				1923, Nov. 2nd wk.				1923, Dec. 3rd wk.															
	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.
42	∞	−	−	−	++	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	Autopsy Jan. 14, 1924. Blood and lungs, 0. Nasal passages, C.+											
	1923, Oct. 1st wk.				1923, Oct. 1st wk.				1923, Oct. 2nd wk.				1923, Oct. 4th wk.				1923, Dec. 1st wk.				1923, Dec. 2nd wk.				1924, Jan. 4th wk.				1924, Mar. 2nd wk.			
140	+	−	−	−	++	−	−	−	H.S.* ++	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−
147	+	−	−	−	++	−	−	−	++	−	−	−	Autopsy Oct. 30, 1923. Blood and lungs, 0. Nasal passages, C. +																			
151	+	−	−	−	++	−	−	−					" " " " " " " 0. " " " +																			

* H.S. indicates hemolytic staphylococcus.

TABLE VII.

Experimental Rabbits. No Snuffles. B. bronchisepticus Present.

Rabbit No.	Date of examination.																																	
	1923, Sept. 3rd wk.				1923, Sept. 4th wk.				1923, Oct. 3rd wk.				1923, Nov. 2nd wk.				1923, Dec. 3rd wk.																		
	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.
17	+	−	−	−	+	−	−	−	∞	−	−	−	∞	−	−	−	++	−	∞	−	Autopsy Jan 11, 1924. Blood and lungs, 0. Nasal passages, Br. ∞															
39	++	−	++	−	++	−	++	−	+	−	++	−	∞	−	−	−	∞	−	−	−	Autopsy Jan. 14, 1924. Blood and lungs, 0. Nasal passages, 0.													
	1923, Oct. 1st wk.				1923, Oct. 1st wk.				1923, Oct. 2nd wk.				1923, Oct. 4th wk.				1923, Nov. 1st wk.				1923, Dec. 2nd wk.				1924, Jan. 4th wk.				1924, Mar. 2nd wk.			
118	++	−	−	−	++	−	−	−	H.S. ++	+	−	−	∞	−	−	−	∞	−	−	−	∞	−	∞	−								
120	++	−	−	−	++	−	+	−	−	−	++	−	∞	−	++	−	++	−	++	−	++	−	−	−								
123	+	−	++	−	++	−	∞	−	H.S. +	−	++	−	∞	−	++	−	++	−	++	−	++	−	++	−								
134	+	−	−	−	++	−	−	−	+	−	−	−									∞	−	−	−	++	−	++	−	++	−	++	−
161									−	−	∞	−	−	−	∞	−	+	−	∞	−	++	−	+	−								
176													+	−	∞	−	++	−	∞	−	++	−	−	−								
178									+	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	−								

TABLE VIII.

Experimental Rabbits. No Snuffles. Bacterium lepisepticum Present.

Rabbit No.	Date of examination.																											
	1923, Sept. 3rd wk.				1923, Sept. 4th wk.				1923, Oct. 3rd wk.				1923, Nov. 2nd wk.				1923, Dec. 3rd wk.											
	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.
12	++	−	−	+	H.S. +	−	−	+	∞	−	−	−	∞	−	−	−	∞	−	−	−	Autopsy Jan. 20, 1924. Blood and lungs, 0. Nasal passages, 0.							
13	++	−	−	++	−	−	−	∞	∞	−	−	+	∞	−	−	−	∞	−	−	−	Autopsy Jan. 17, 1924. Blood and lungs, 0. Nasal passages, L. +							
29	+	−	−	−	+	−	−	+	∞	−	−	−	++	−	−	−	++	−	−	∞	Autopsy Jan. 14, 1924. Blood and lungs, 0. Nasal passages, L. ∞							
	1923, Oct. 1st wk.				1923, Oct. 1st wk.				1923, Oct. 2nd wk.				1923, Oct. 4th wk.				1923, Nov. 1st wk.				1923, Dec. 2nd wk.				1924, Jan. 4th wk.				1924, Mar 2nd wk.			
127	+	−	−	+	+	−	−	∞	+	−	−	∞	++	−	−	++	Autopsy Nov. 7, 1923. Blood 0. Nasal passages, L. ∞											
128	++	−	−	−	++	−	−	−	++	−	−	−	∞ ∞	−	−	−	−	−	−	∞	∞	−	−	+	++	−	−	++	++	−	−	++
129	+	−	−	−	++	−	−	−	++	−	−	−	∞ ∞	−	−	−	++	−	−	−	++	−	−	++	++	−	−	++	++	−	−	++
132	−	−	−	∞	+	−	−	∞	+	−	−	∞ ∞	++	−	−	+	++	−	−	++	Autopsy. No cultures.										
135	+	−	−	++	+	−	−	++	+	−	−	∞ ∞	++	−	−	++	++	−	−	∞	++	−	−	∞	∞	−	−	−	∞	−	−	−
174													++	−	−	+	∞	−	−	−	Autopsy Nov. 14, 1923. Blood and lungs, 0. Nasal passages, C. +							
175													+	−	−	∞	++	−	−	∞	+	−	−	−	−	−	−	−	−	−	−	−

TABLE IX.

Experimental Rabbits. Snuffles. Bacterium lepisepticum Present.

Rabbit No.	Date of examination.																																			
	1923, Sept. 3rd wk.				1923, Sept. 4th wk.				1923, Oct. 3rd wk.				1923, Nov. 2nd wk.				1923, Dec. 3rd wk.																			
	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L.	C.	Int. group.	Br.	L	C.	Int. group.	Br.	L	C.	Int. group.	Br.	L	C.	Int. group.	Br.	L
7	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	+	−	−	∞	Autopsy Jan. 16, 1924. L. ∞				Blood and lungs, 0.				Nasal passages,							
8	−	−	−	∞	H.S. +	−	−	∞	−	−	−	∞	+	−	−	∞	−	−	∞	+	Autopsy Jan. 16, 1924. L. ∞				Blood and lungs, 0.				Nasal passages,							
9	−	−	−	∞	H.S. +	−	−	∞	+	−	−	∞	+	−	−	∞	+	−	−	∞	Autopsy Jan. 16, 1924. L. ++				Blood and lungs, 0.				Nasal passages,							
11	−	−	∞	∞	H.S. +	−	++	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞																	
15	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞																	
21	−	−	−	∞	++	−	−	∞	−	−	−	∞	−	−	−	∞	+	−	−	∞	Autopsy Dec. 26, 1923. L. ++				Blood and lungs, 0.				Nasal passages,							
24	−	−	−	∞	+	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	Autopsy Jan. 15, 1924. L. ∞				Blood and lungs, 0.				Nasal passages,							
31	++	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	−	−	−	∞	Autopsy Dec. 29, 1923. L. ∞				Blood and lungs, 0.				Nasal passages,							
35	−	+	−	++	+	−	−	∞	++	−	−	∞	−	−	−	∞	−	−	−	∞	Autopsy Jan. 22, 1924. L. ∞				Blood and lungs, 0.				Nasal passages,							
37	−	−	−	∞	−	−	∞	∞	−	−	−	∞	−	−	−	∞	Autopsy. No cultures.																			
38	−	−	−	∞	++	−	−	∞	++	−	−	∞	−	−	−	∞	−	−	−	∞																	

In brief, then, of thirty-two normal rabbits in this series, 58 per cent showed *Bacterium lepisepticum* and 53 per cent showed *Bacillus bronchisepticus;* while 12 per cent showed neither *Bacterium lepisepcum* nor *Bacillus bronchisepticus* during the entire period of observation. In all eleven rabbits with clinical snuffles *Bacterium lepisepticum* was the predominating organism.

DISCUSSION.

The bacteriological study of the rabbit stock described in this and the preceding papers[1,3] has supplied us with data on the frequency and distribution of certain respiratory infections among the stock of The Rockefeller Institute. The rabbits examined were taken from nearly every room in the building and thus present a general index of the prevailing conditions.

We have estimated on the basis of these data that from 35 to 45 per cent of the entire stock is affected with clinical snuffles. This average percentage is maintained throughout the year by the continual addition from outside sources of partly healthy and partly infected stock obtained from the dealers. On the other hand, pneumonia and septicemia are relatively infrequent and no severe epidemic of respiratory disease has prevailed among the rabbits for some years.

From the nasal passages of all animals affected with clinical snuffles, *Bacterium lepisepticum* has been cultured during life or at autopsy. In the great majority of cases, this organism greatly outnumbered all others and was often isolated in pure culture. *Bacterium lepisepticum* may be cultured during life or at autopsy from 30 to 40 per cent of normal rabbits upon arrival at the Institute, and from 60 to 70 per cent after they have been quartered in the animal house for a period of time. Therefore, at least 70 per cent of the stock were carriers of *Bacterium lepisepticum.*

The bacteria accompanying this organism consisted of relatively a simple and constant group; in 80 per cent of the rabbits, the *catarrhalis* and other Gram-negative cocci were found, and in 40 per cent *Bacillus bronchisepticus* was also present, while the Gram-positive cocci

[3] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837.

and intestinal group of organisms found near the external nares occurred in 10 to 15 per cent of the aniamls.

Bacillus bronchisepticus was found equally in 30 to 40 per cent of normal and of snuffles rabbits.

The fact that *Bacterium lepisepticum* is constantly associated with rabbit snuffles in the rabbits studied and is present in the nasal passages of so many other rabbits has led us tentatively to consider it as the bacterial incitant of that disease and to investigate further its pathogenic properties as a step toward reproducing snuffles experimentally.

SUMMARY.

The bacteria constituting the nasal flora of rabbits at The Rockefeller Institute may be enumerated as follows in the order of their frequency: (1) *Micrococcus catarrhalis* group, 80 per cent; (2) *Bacterium lepisepticum*, 70 per cent; (3) Gram-negative cocci which ferment dextrose, lactose, saccharose, maltose, and mannite, but not salicin and *Bacillus bronchisepticus*, 40 per cent; (4) Staphylococci, streptococci, and various intestinal bacilli which probably are localized at the external nares, 10 per cent.

Bacterium lepisepticum is the predominating organism in the nasal passages of the rabbits affected with snuffles.

We wish to thank Mr. Caspar Burn for assisting with the technical work involved in these experiments.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

IV. The Effect of Selective Breeding on Host Resistance.

By LESLIE T. WEBSTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Previous studies on mouse typhoid have shown us that in an experimentally controlled community individuals differ in resistance to infection and that this resistance contains determinable elements of a non-specific nature.[1] It seemed probable, therefore, that the non-specific factors could be influenced by modifications in heredity or environment and that the resulting variations would affect the general resistance of the individual. In the following experiments we have undertaken to control the heredity by the selective breeding of mouse typhoid survivors and have tested the effect of this mode of selection on resistance to the specific infection, mouse typhoid, and to a non-specific poisonous substance, mercury bichloride.

The Rockefeller Institute mice have been strictly inbred for 5 or 6 years. Prior to that time various strains were added occasionally and incorporated with the stock. The present stock consists, therefore, of inbred hybrid mice raised under conditions that have been identical for a period of years.

Any group of these mice infected *per os* with a certain dose of mouse typhoid bacilli will die in a predictable and characteristic manner represented by our standard mortality curve.[2] Consequently, this Rockefeller Institute strain of mice and the culture of *Bacillus pestis caviæ* designated as M. T. II may be employed with a fair degree of accuracy to determine variations either in properties of microbe or of host.

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 129.

[2] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

In the following experiments the heredity factor of the host has been altered but all other conditions,—food, surroundings, age, and weight of the mice, media, incubation period, and dose of the bacterial culture,—have been kept constant.

Selective Breeding and Mouse Typhoid Infection.

Experiment 1.—The first experiment was planned to determine the effect of selective breeding on resistance to mouse typhoid by infecting mice, breeding the survivors, and infecting their offspring for a successive number of generations.

Series 1.—100 mice weighing 16 to 18 gm. were given by stomach tube about 4,000,000 mouse typhoid bacilli,[3] Strain M. T. II, from an 18 hour broth culture. 2 months later, 76 of these mice were dead. Ten survivors, males and females, which showed no mouse typhoid bacilli in blood or feces and no specific agglutinins, were taken to The Institute stock breeding room and mated. 3 months later, fourteen offspring of these mice were available.

These second generation mice weighing 16 to 18 gm. each, together with twenty-five control mice of similar weight from the general unselected stock, were given *per os* about 4,000,000 bacilli of the M. T. II strain diluted from an 18 hour broth culture. Fatal cases were then recorded, autopsied, and cultured for the specific organism.

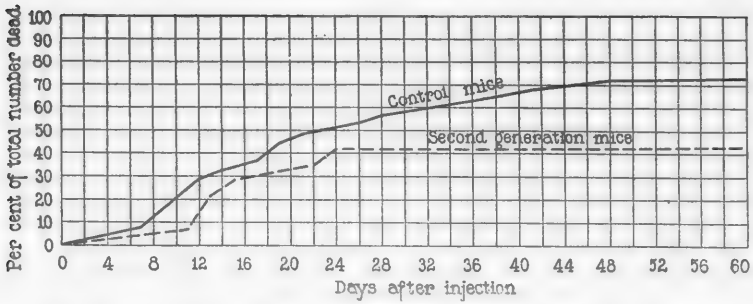
Text-fig. 1 records the mortality of each group of mice and shows that while 72 per cent of the unselected controls were dead at 8 weeks, only 42 per cent of the selected stock had succumbed. This latter group, therefore, was somewhat more resistant to mouse typhoid infection than a similar group not so selected.

After a suitable period of time, six of the second generation survivors, free from mouse typhoid bacilli in blood and feces and from specific agglutinins, were mated in the breeding room and a third generation was raised.

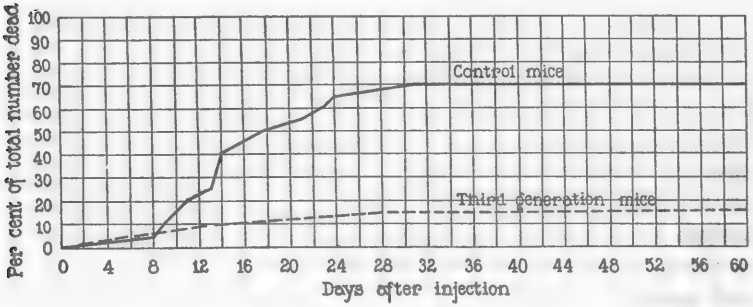
3 months later, twenty-one of these mice and twenty-one controls varying in weight from 16 to 18 gm. were given the same mouse typhoid strain *per os*—3,000,000 bacteria per mouse. Autopsy and bacteriological procedures were repeated and the mortality ratio to time was again plotted (Text-fig. 2).

In this experiment (Text-fig. 2), the mortality of the controls reached 70 per cent, while that of the third generation selected stock

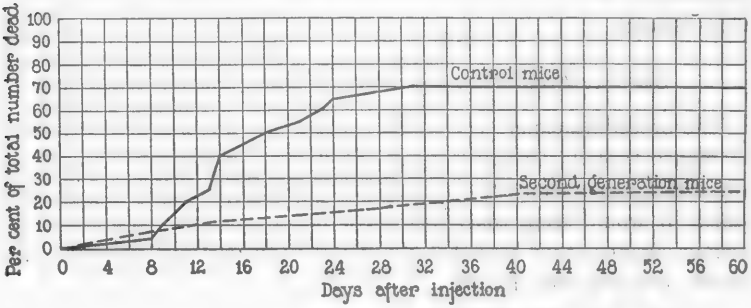
[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.



TEXT-FIG. 1. Series 1. Effect of selective breeding on the resistance of mice to mouse typhoid infection. Second generation.



TEXT-FIG. 2. Series 1. Effect of selective breeding on the resistance of mice to mouse typhoid infection. Third generation.



TEXT-FIG. 3. Series 2. Effect of selective breeding on the resistance of mice to mouse typhoid infection. Second generation.

was only 15 per cent. Furthermore, it may be emphasized that the third generation group proved to be more resistant than the second generation group (compare Text-figs. 1 and 2).

Series 2.—This experiment has been conducted in duplicate in the following manner.

Several mice, male and female, which had survived in turn two lethal doses of paratyphoid-enteritidis cultures and a lethal dose of mercury bichloride,[1] were mated.

3 months later, sixteen offspring from these mice, having reached the size of 16 to 18 gm., were assembled with the third generation and control groups as described in the preceding paragraph and inoculated with a similar dose of the same culture. The subsequent procedures were the same as those previously carried out.

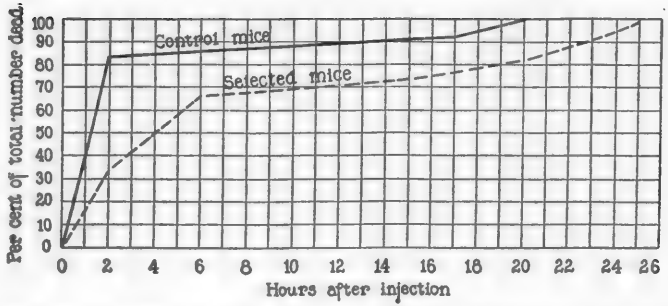
The mortality of this second generation group proved to be 24 per cent; that of the controls was 70 per cent (Text-fig. 3).

These experiments have shown that by the repeated selection and breeding of mice which have survived a native disease, such as mouse typhoid, it is possible to enhance progressively the resistance of their offspring to that disease. Specific immunization *in utero* from the mother's blood or *post partem* immunization from the mother's milk, or from bacilli that she might discharge with the feces, were excluded as the active factors by the examination of the blood and stools of each parent. On the other hand, the rise in the degree of protection with each generation and the non-specific as well as specific character of the resistance displayed, such as is shown in the next experiment, indicate that the greater resistance is dependent on hereditary factors.

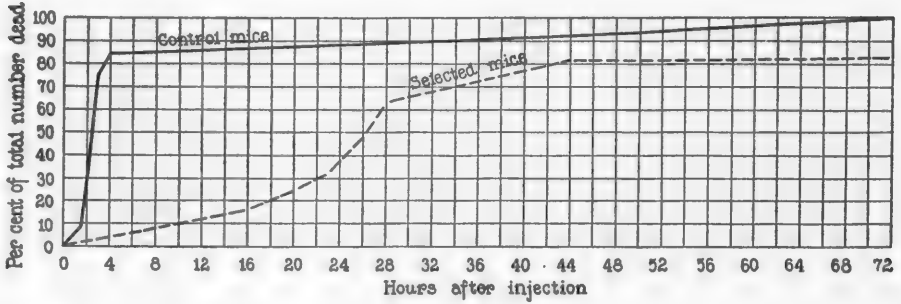
Selective Breeding and Mercury Bichloride Intoxication.

The preceding experiments have shown that the selective breeding of mouse typhoid survivors progressively increases the resistance of succeeding generations to this infection. But in the light of previous work,[1] it would seem that this increase should operate against deleterious agents other than mouse typhoid infection and contain elements of non-specific nature.

To test this view further, we employed a chemical substance, bichloride of mercury, and have compared the resistance of selected and control mice to this chemical.



TEXT-FIG. 4. Effect of selective breeding on the resistance of mice to mercury bichloride poisoning. Dose 0.0033 gm. per mouse.



TEXT-FIG. 5. Effect of selective breeding on the resistance of mice to mercury bichloride poisoning. Dose 0.0025 gm. per mouse.

Experiment 2:—

Test A: Twelve mice, the second litter of second generation typhoid-resistant mice (see Experiment 1), together with twelve unselected normal control mice of similar weight, 16 to 18 gm. each, were given *per os* in ½ cc. volume of distilled water 0.0033 gm. of mercury bichloride. The specially bred and control mice were injected alternately.

Text-fig. 4 plots the duration of life of each series and shows that the specially bred typhoid-resistant mice were somewhat more resistant to the bichloride than the control mice.

Test B: Twelve mice, the second litter of third generation typhoid-resistant mice (see Experiment 1), together with twelve unselected normal control mice of similar weight, 16 to 18 gm., were given *per os* 0.0025 gm. of mercury bichloride in ½ cc. of distilled water. The specially bred and control mice were injected alternately.

Text-fig. 5 plots the duration of life of each series and shows that the selected mice were considerably more resistant to the bichloride than control mice.

From these tests it may be concluded that the selectively bred typhoid-resistant mice are more resistant to a lethal dose of mercury bichloride than are unselected control mice.

DISCUSSION.

The most direct experimental evidence relating to the inheritance of resistance to infection has been reported by botanists. Biffen's experiments with wheat,[4] those of Barrus and of McRostie with beans,[5,6] Parker's with oats,[7] and Salaman and Lesley's work with potatoes[8] constitute part of the evidence which led Brooks to say in a general article concerning disease resistance in plants:[9] "It is a matter of common observation to cultivators that different varieties of the

[4] Biffen, R. H., *J. Agric. Sc.*, 1912, iv, 421.
[5] Barrus, M. F., *Phytopath.*, 1918, viii, 589.
[6] McRostie, G. P., *Phytopath.*, 1919, ix, 141.
[7] Parker, J. H., *J. Am. Soc. Agron.*, 1920, xii, 23.
[8] Salaman, R. N., and Lesley, J. W., *J. Genetics*, 1923, xiii, 177.
[9] Brooks, F. T., *Brit. Med. J.*, 1922, ii, 964.

same cultivated plant show marked differences in the power of resisting certain parasitic diseases. . . . . There must clearly be some constitutional or genetic difference between these closely related plants to account for such phenomena."

Evidence for the inheritance of resistance in laboratory animals is not abundant. Leo Loeb, in one of his studies on the inheritance of cancer in mice, makes the statement that "the resistance to disease in general, is also hereditarily transmitted."[10] Guyer and Smith[11] and Learmonth[12] have recently published experiments which indicate that the ability of rabbits or guinea pigs to form typhoid agglutinins may be increased by selective breeding. Wright and Lewis[13] in their paper "Factors in the resistance of guinea pigs to tuberculosis, with especial regard to inbreeding and heredity" state that:

"Marked differences in resistance were found among the number of inbred families of guinea pigs.

"The high resistance of one of these families was transmitted by each sex to the offspring of each sex in crosses with other inbred families."

Finally, there exists a great deal of empirical evidence and tradition relating to this question as it affects man and his reaction to infectious disease. The hypersusceptibility of the savage to the ailments of civilized man, and the greater severity of epidemics among isolated peoples, have been explained on the basis of natural selection and breeding of survivors.[14,15]

Cummins[16] and Bushnell[17] have attempted to explain such variations in susceptibility to tuberculosis on the basis of a specific acquired immunity, but the ultimate solution of the problem must await the accumulation of more experimental facts.

[10] Loeb, L., *Am. Naturalist*, 1921, lv, 510.

[11] Guyer, M. F., and Smith, E. A., *J. Infect. Dis.*, 1923, xxxiii, 498.

[12] Learmonth, J. R., *J. Hyg.*, 1923, xxii, 100.

[13] Wright, S., and Lewis, P. A., *Am. Naturalist*, 1921, lv, 20.

[14] Pearson, K., and Elderton, E. M., *Biometrika*, 1913 ix, 320.

[15] Bauer, J., Die konstitutionelle Disposition zu inneren Krankheiten, Berlin, 1917.

[16] Cummins, S. L., *Internat. J. Pub. Health*, 1920, i, 137.

[17] Bushnell, G. E., A study in the epidemiology of tuberculosis with especial reference to tuberculosis of the tropics and of the negro race, New York, 1920.

The experiments reported in this paper were performed under conditions which reduced the possibility of specific acquired immunity to a minimum and indicate, therefore, that in mice, host resistance contains important hereditary factors both specific and non-specific in nature and that the degree of this resistance in a given population may be enhanced by selective breeding.

CONCLUSION.

If mice surviving a lethal dose of mouse typhoid bacilli are inbred consecutively for a number of generations, the resulting offspring become progressively more resistant to mouse typhoid infection and to mercury bichloride intoxication than similar control mice not so selected.

THE CONCENTRATION OF THE PLASMA PROTEINS IN NEPHRITIS.

By G. C. LINDER, M.D., C. LUNDSGAARD, M.D., AND D. D. VAN SLYKE, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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In spite of the large number of observations which have been made by investigators during the past 60 years, there are still differences of opinion as to the changes which occur in the protein concentration in the plasma in nephritis and allied conditions. In this paper is reported a series of observations on the albumin and globulin concentration in the plasma of patients with nephritis, some of whom have been under observation for prolonged periods. An attempt is made to determine the relationship of these changes to edema and proteinuria, and to the clinical type of the disease.

In 1836 Bright (1), describing those cases of edema and albuminuria since called by his name, wrote: "If the blood is drawn, it is often buffed, or the serum is milky and opaque; and nice analysis will frequently detect a great deficiency of albumen." This deficiency of total serum protein has since been repeatedly noted. After Hammarsten (2) in 1878 introduced a method for salting out the globulins, Csatáry (3) noted that the deficiency affected the serum albumin more than it did the globulins, so that the albumin: globulin ratio, normally 1.5 or 2.0, fell below 1. Csatáry quotes Mya and Viglezio (4) as having already noted this fall in the A/G ratio in nephritis, diabetes, and pneumonia. With the progressive refinement of methods, the effects of various factors on the serum proteins have been the object of many investigations (5–13) which have been reviewed up to 1916 by Rowe (10).

In 1912 and 1913 Epstein (14, 15) published analyses of the plasma proteins in a number of diseases. A series of normal plasmas showed total protein contents of 6.4 to 8.1 per cent, with A/G ratios of 1.2 to 2.3. He found a very marked reduction in the total concentration and the A/G ratio in chronic parenchymatous nephritis, but very little change in chronic interstitial nephritis. In 1917 (16) and 1922 (17) he found that these changes were extreme in those cases which could be differentiated from true glomerulonephritis, and for which Friedrich Müller (18) in 1905 proposed the name nephrosis. He attributed the edema to the fall in osmotic pressure of the plasma consequent upon its low protein content.

Kahn (19), on the other hand, in 1920, failed to find low figures for the plasma proteins in twenty-three cases of chronic parenchymatous nephritis, although sixteen of them had great edema.

Method Employed in This Investigation.—Our determinations were made upon oxalated plasma. The blood was drawn from the antecubital veins with as little stasis as possible, the patient having breakfasted 2 to 3 hours before, and remaining in bed until after the bleeding. For the analysis of the proteins the micro Kjeldahl method of Howe (20) was employed. Analyses were made in duplicate, sometimes in triplicate. Satisfactory checks were obtained in all the results reported.

In Table I are shown the results obtained from the analysis of seven normal plasmas. With one exception, L.D., they agree with the older figures from the literature, and with the recent ones of Epstein (14, 15), but they are a little lower than the figures Rowe obtained with the refractometer. In a certain number of cases the proteins were estimated both by Howe's method and by means of the refractometer. The results are presented in Table II and show definitely higher figures for the refractometric method. The first determination on Subject L.D. showed that occasionally wider variations may occur in healthy people than is commonly supposed.

Classification of the Patients Studied.—In grouping our patients with nephritis we have adopted as a basis the classification and terminology of Volhard and Fahr (21). They are therefore divided into three groups; namely, nephrosis, diffuse glomerulonephritis, (divided into two subgroups), and nephrosclerosis.

The characteristic features of the nephrosis group are the tendency to extreme edema and large serous effusions, the passage of a scanty urine of high specific gravity containing much protein and many casts, but no blood, the absence of anemia, of increased blood pressure, of retinal hemorrhages and exudates, and of any considerable or permanent increase of non-protein nitrogen in the blood. The blood urea nitrogen is sometimes abnormally low in chronic cases. This group together with the nephrotic subgroup of glomerulonephritis includes all the cases formerly classed together under the heading of "large white" kidneys. Histologically there is conspicuous lipoid degeneration of the tubules with a deposit of lipoids in the interstitial tissue; some of this lipoid material is doubly refracting. The changes in the glomeruli are slight and most of the glomeruli remain unaffected. There are no vascular lesions.

In the group of glomerulonephritis the characteristic features are raised blood pressure; protein, blood, and casts in the urine; and more or less severe anemia. Edema frequently occurs but may be absent. In acute cases which are effectively

TABLE I.

Protein Determinations in the Plasma of Normal Individuals.

Case.	Albumin.	Globulin.	Total protein.	Ratio of albumin to globulin.	Non-protein nitrogen.
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*		*gm. per 100 cc.*
L. R.	4.51	2.45	6.96	1.8	0.017
L. D.					
Jan. 24, 1923.	3.36	2.26	5.62	1.5	0.030
Mar. 12, 1923.	4.08	2.76	6.84	1.5	0.017
S.N.	4.10	2.60	6.70	1.6	0.025
N. L.	4.11	2.89	7.00	1.4	0.018
S. N.	4.22	2.80	7.02	1.5	0.020
B. S.	4.90	2.55	7.45	2.0	0.014
O. P.	3.62	2.60	6.22	1.4	0.018
Average.....	4.11	2.61	6.73	1.6	

TABLE II.

Comparison of Determinations of the Plasma Proteins by Kjeldahl Method and Refractometric Method.

Case.	Total protein by Kjeldahl.	Total protein by refractometer.
	gm. per 100 cc.	*gm. per 100 cc.*
M. L.	7.5	7.0
H. D.	5.6	6.4
L. Y.	4.1	5.1
K. G.	6.9	8.3
H. L.	7.1	7.6
R. H.	7.0	7.1
R. N.	7.3	8.4
K. R. N.	7.6	8.4
K. F. N.	6.6	7.6
Average.....	6.6	7.3

Classification of Nephritis

	Characteristic symptoms.	Symptoms characteristically absent.
NEPHROSIS.	EDEMA.	HYPERTENSION.
Stage I. (*a*) Acute.	Great edema and serous effusions.	Retinitis.
(*b*) Transitional.	Serous effusions and slight edema.	Hematuria.
Stage II.	Slight edema and small effusions with tendency to relapse.	Anemia.
Stage III.		
FOCAL NEPHRITIS.	HEMATURIA.	HYPERTENSION. EDEMA.
DIFFUSE GLOMERULONEPHRITIS.	HYPERTENSION. CARDIOVASCULAR HYPERTROPHY.	
Stage I. Acute.	Transient.	
Stage II. (*a*) Glomerulotubular.	Slight.	
(*b*) Vascular-interstitial.	Moderate.	
Stage III. (*a*) Glomerulotubular.	Slight or moderate.	
(*b*) Vascular-interstitial.	Extreme.	Renal edema.
NEPHROSCLEROSIS.	EXTREME HYPERTENSION AND CARDIOVASCULAR HYPERTROPHY.	
Stage I. (Benign.)	Extreme (180–220 mm.).	Anemia, hematuria, ren edema.
Stage II. (Malignant.)	Very extreme (200–300).	Renal edema.

Volhard and Fahr).

Renal function.			Other features.
Blood urea nitrogen.	Phthalein output.	Dilution and concentration test.	
Slight increase.	Moderately low.	Dilution impaired; concentration good.	REDUCTION OF PLASMA PROTEIN WITH TYPICALLY LOW A/G RATIO.
Normal or low.	Normal.	Normal.	
Normal or low.	Normal.		
Increased.	?	Concentration impaired; dilution retained.	
NORMAL.	NORMAL.	NORMAL.	
	REDUCED.		LOW PLASMA PROTEINS (Not typical of III (*b*)). ANEMIA.
Transient increase.	Reduced.	Temporary impairment; dilution more impaired.	Edema (Not typical of II (*b*), or III(*b*)).
Usually moderately increased.	Normal or moderately reduced.	Normal or hyposthenuria; concentration more impaired; dilution well preserved.	Retinitis.
Moderately increased.	Greatly reduced or absent.	Isosthenuria. Dilution affected last.	Uremia.
Extremely increased.			Marked retinitis.
			AGE AND HISTORY. RETINAL HEMORRHAGES AND ARTERIOSCLEROSIS.
Normal.	Normal.	Normal or hyposthenuria.	
Increased, often greatly.	Reduced.	Isosthenuria.	Marked retinitis.

treated at an early stage healing is the rule, but under less fortunate circumstances the condition passes into a subacute or chronic stage in which complete recovery is no longer possible. In these persistent cases retention of non-protein nitrogen in the blood, impairment of response to tests of renal function, and the condition of isosthenuria sooner or later supervene, and retinitis and uremia are frequent.

Volhard subdivides these cases into two subgroups, the glomerulotubular, or nephrotic, and the vascular-interstitial.

In the glomerulotubular subgroup the increase of blood pressure and cardiac hypertrophy are moderate, and the tendency to edema and serous effusions is considerable; post mortem a "large white" kidney is usually found showing histologically well marked lesions in the glomeruli and the tubules. Death from intercurrent infections is frequent, and of the patients who escape this fate many in the course of time lose their tendency to renal edema, and develop greater cardiovascular changes and renal insufficiency, associated with a secondary contraction of the kidneys. In a few of them, however, edema of renal origin persists even during the terminal stage.

In the vascular-interstitial subgroup the increase of blood pressure and cardiac hypertrophy are conspicuous and the tendency to edema of purely renal origin is slight and frequently absent. In the kidneys there are lesions in the glomeruli and the smallest arteries, with interstitial infiltration, and in long standing cases the kidneys are white and contracted, the "small white" kidney. This type occurs less frequently. The onset is often insidious and the victim frequently comes under medical care only when his eyesight fails from retinitis or from weakness or vomiting when uremia is imminent.

The nephrosclerosis group comprises those cases in which the primary lesion is in the vessels of the kidneys, although at a later stage the picture may be complicated by heart failure and a true glomerular inflammation. The dominant clinical features are the great increase in blood pressure and the great cardiac hypertrophy. In the early stages the signs of renal disease may be completely lacking, proteinuria may be absent and is often scanty, and renal function is normal. In the final stages inflammatory glomerular lesions occur and true uremia very frequently supervenes.

Our series included fourteen cases of glomerulonephritis of which eleven were of the glomerulotubular variety. These all showed edema at some time during the period of observation; its degree varied from slight puffiness of the face and pitting over the shins to extreme anasarca with serous effusions. There were three cases of the vascular-interstitial type, and on two of these postmortem examinations were made. In both we found the diffuse fatty lesion of the smallest arteries of the kidneys and other organs which Evans (22) has described as "diffuse hyperplastic intimal sclerosis." Evans finds

diffuse hyperplastic sclerosis in the intimate vasculature not only of the kidney but of the spleen, pancreas, or other organs in all cases in which at autopsy there is considerable cardiac hypertrophy in the absence of primary heart or lung disease. He reports cases in which glomerular and interstitial inflammation without this diffuse vascular lesion produced extremely contracted kidneys but these cases showed no elevation of blood pressure during life and no cardiac hypertrophy at autopsy. He considers that marked elevation of the blood pressure is the clinical expression of diffuse hyperplastic intimal sclerosis.

There were five cases of nephrosis among our patients. Three of them were acute cases in which complete recovery occurred, but in one the condition was very mild and another was studied only during the stage of recovery. The remaining two were chronic cases. Five cases of nephrosclerosis were studied, and two cases of functional proteinuria, which presented none of the symptoms of renal disease and gave normal responses to tests of renal function.

The details of the patients are presented in Tables III to VII and Charts 1 to 5. They show the course of the main symptoms and give the figures obtained for the concentration of proteins in the plasma. The results of some of the tests of renal function are included in the tables. The tests we have commonly employed have been:

1. The determination of the blood urea and non-protein nitrogen, and creatinine.
2. The urea index (Austin, Stillman, and Van Slyke (23)).
3. The urea concentration test (Maclean and de Wesselow (24)).
4. The iodide excretion test (Linder (25)).
5. The "phthalein" excretion test.
6. The dilution and concentration tests (modified from Strauss (26)).

Autopsies were obtained upon two of the patients who died. The important findings are given below.

Case 12.—The heart weighed 450 gm. The kidneys were not contracted and weighed 290 gm. together. The capsule was easily stripped from a finely granular surface. On section the differentiation of the cortex and medulla was completely obscured. A few epithelial crescents were found but lesions in the glomeruli were inconspicuous. There was moderate interstitial inflammation. Typical diffuse hyperplastic intimal sclerosis was found in the kidneys, spleen, pancreas, and bladder. Tubular degeneration was advanced. A *Staphylococcus aureus* septicemia was present.

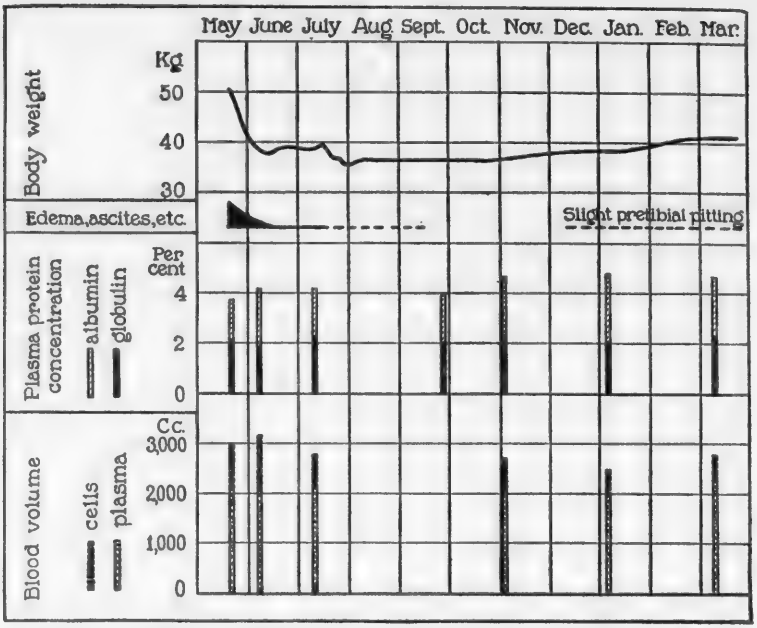

CHART 1. Case 1. (Table III.) Chronic glomerulonephritis, nephrotic type. In the upper sections are shown the changes in body weight, edema, and concentration of the plasma proteins. In the bottom section the results of plasma volume determinations are recorded; these are discussed in the following paper (Linder, G. C., Lundsgaard, C., Van Slyke, D. D., and Stillman, E., *J. Exp. Med.*, 1924, xxxix, 921).

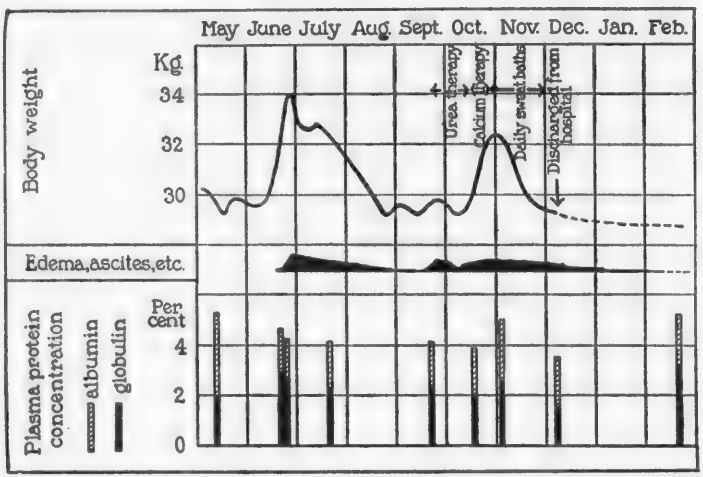

CHART 2. Case 2. (Table III.) Chronic glomerulonephritis, nephrotic type. No plasma volume determinations were made.

Case 14.—The heart weighed 530 gm. The kidneys were contracted and weighed 190 gm. together. The capsule was thick and adherent; the surface was reddish white and finely granular. On section the cortex was narrow but quite distinct from the medulla. Glomerular crescents were present but not numerous. Fatty degeneration of the tubules and interstitial inflammation were marked. Diffuse hyperplastic sclerosis was found in the kidney, spleen, pancreas, and prostate.

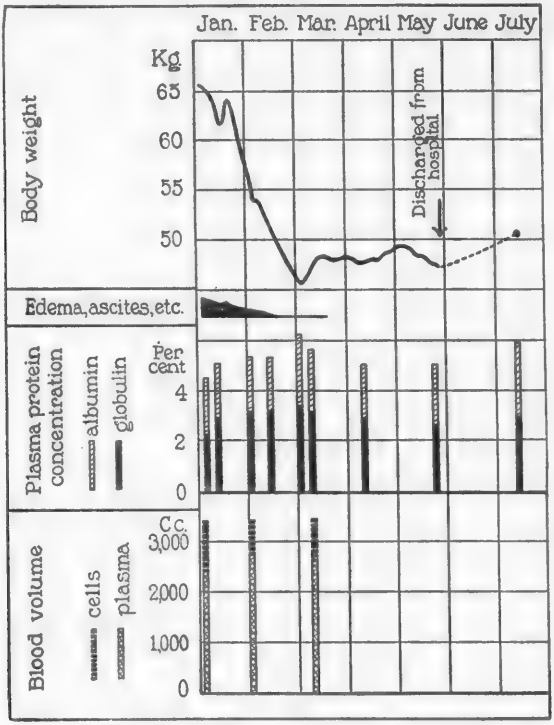

CHART 3. Case 8. (Table III.) Subacute glomerulonephritis, nephrotic type.

Analysis of Results.—Glomerulonephritis, Nephrotic Type: Eleven cases (Table III). Four cases were studied in considerable detail for periods varying from 9 to 19 months, and seven were studied less systematically but the results obtained from them help to complete the picture.

The protein content of the plasma was less then 5.5 per cent in all the chronic and subacute cases in which the disease was or had recently been active. The patients showing higher figures were No. 9, a convalescent from acute glomerulonephritis, who had at the beginning of his illness edema, which had disappeared long before the

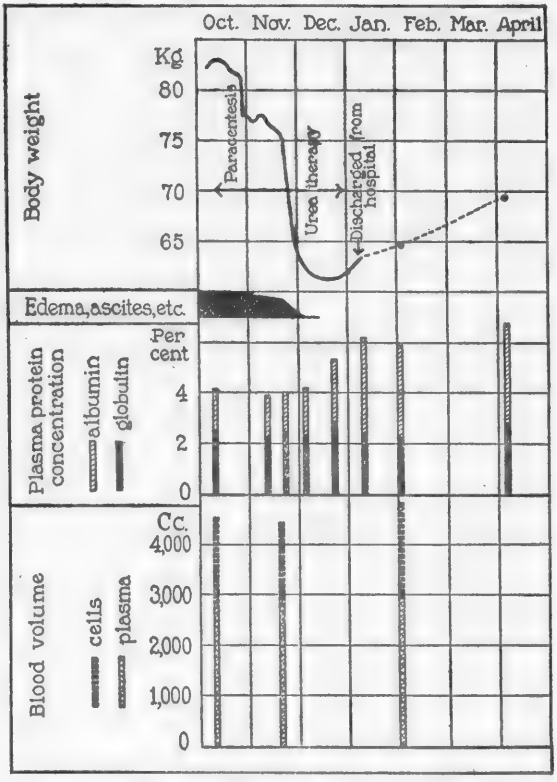

CHART 4. Case 15. (Table V.) Acute nephrosis.

observations were made on his plasma, and Nos. 4 and 7, chronic cases in a stage of arrest with considerable permanent impairment, in which the proteinuria had fallen to a small amount. In the remaining eight cases protein contents of 5.5 to 3.5 per cent were observed. All of these had had some degree of edema during their illness, but many

low concentrations were found at times when no edema was present, and no constant connection between changes in the proteins and changes in the degree of edema was defined. In some patients (Nos. 1, 2, 5, and 8) the lowest figures were found when the edema was increasing or at its greatest. In No. 1, 3 months after the subsidence of edema, the protein concentration was practically unchanged (Chart

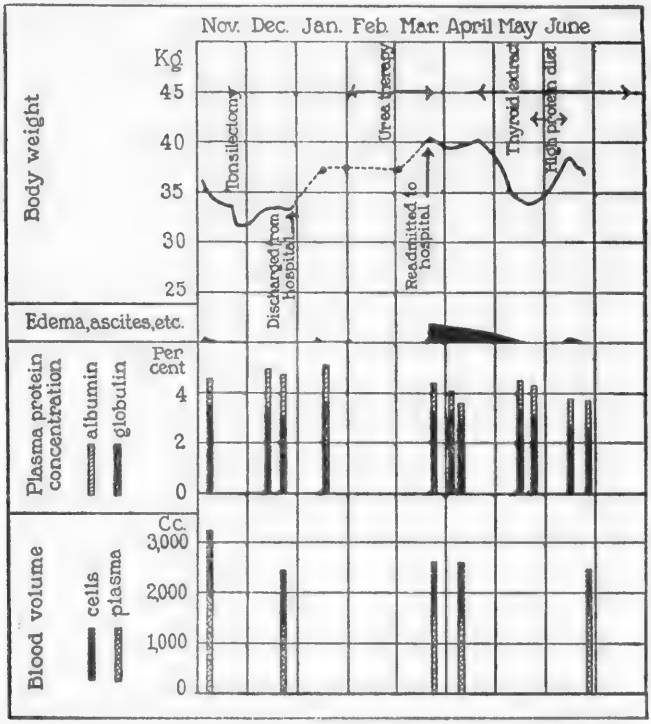

CHART 5. Case 16. (Table V.) Chronic nephrosis.

1). In No. 3 the protein which initially was but moderately reduced fell to a lower level after the edema had gone. In No. 2 there occurred a persistent decrease in the concentration of the plasma proteins during the period from May to October in spite of the development of edema in June and its elimination in July and August. The increase of edema in November was associated with a rise in the plasma pro-

TAB

Cases of Glomerulonephri

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10D}{B\sqrt{VW}}$.	Phthalein 2 hrs.	Body weig
	1922		*gm. per liter*		*per cent*	*kg.*
No. 1 (J. D.). Male.	May 20.	140/76	0.21	45	45	50.4
14 yrs.	June 7.	120/80	0.28	43	68	39.0
May, 1922.	July 10.	124/84	0.19			38.8
	Sept. 27.	124/74	0.20	43		36.1
	Nov. 2.	116/70	0.26	28		36.8
	1923					
	Jan. 5.	120/66	0.20	37		38.2
	Mar. 9.	120/66	0.15	38		41.6
	Oct. 26.	130/70	0.23	36	50	42.6
	Nov. 24.	105/55	0.53	10		45.3
	" 26.	130/66	0.55			42.4
	Dec. 1.	122/55	0.69			40.8
	1922					
No. 2 (H. L.). Male.	May 11.	160/110	0.18	49	41	29.9
10 yrs.	June 20.	132/90	0.27	29	46	32.2
Apr., 1921.	" 23.	132/94	0.40	20		32.8
	July 20.	130/90	0.36	25	42	31.6
	Sept. 21.	130/92	0.29	25	39	29.3
	Oct. 17.	115/85	0.86*	25		30.1
	Nov. 3.	120/90	0.44	13		31.5
	Dec. 7.	134/94	0.32	20	30	29.1
	1923					
	Feb. 20.	150/?	0.46			28.1

* During period of urea feeding maximum urea in urine 2.1 per cent.

hrotic Type.

Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
			Albumin.	Globulin.	Total.	A/G ratio.	
	gm. per 24 hrs.						
ral edema. Ascites. ydrothorax.	3.2	+++	1.6	2.1	3.7	0.76	Chronic glomerulonephritis. Chart 1.
slight edema.	4.0	+++	2.3	1.8	4.1	1.28	After disappearance of the edema the case was remarkable for persistent hematuria. The impairment of renal function was moderate, but changes in the fundi appeared during 1923.
bial pitting.	8.5	+++	1.93	2.13	4.06	0.91	
	3.9	+++	2.00	1.93	3.93	1.03	
dema.	4.0	++	2.23	2.29	4.52	0.97	
"	4.4	++	2.66	2.00	4.66	1.32	Urine urea after urea feeding 2.2 per cent Aug. 27, 1922.
"	7.1	+	2.27	2.32	4.59	0.97	Iodide excretion 40 per cent Feb. 20, 1923.
slight pretibial pitg.	12.8	++	2.03	2.34	4.37	0.87	Readmitted Nov. 24, 1923, with acute streptococcal sepsis; died on Dec. 4, 1923.
dema.	5.1	+	1.61	2.06	3.67	0.78	
"	4.4	+	1.72	2.32	4.04	0.74	Autopsy: large white kidneys; moderate glomerulonephritis with marked tubular degeneration; no vascular sclerosis. Heart: 290 gm.
t edema.			1.69	2.38	4.02	0.73	
nt.	3.9	(+)	3.3	1.95	5.25	1.7	Chronic glomerulonephritis. Chart 2.
puffy.	3.1	+	1.65	2.94	4.59	0.56	No retinal disease.
es. Hydrothorax. dema.	3.8	+	1.58	2.68	4.26	0.59	From June 8 to 29 there was a period of increasing edema, the weight rising to 34 kilos.
easing edema, etc.	2.8	+	1.83	2.25	4.08	0.81	
t edema and ascites.	6.6	+	1.82	2.32	4.14	0.78	From July to Aug. this edema was eliminated. Weight 29 kilos.
easing ascites.	5.8	+	1.86	1.96	3.82	0.95	
es and hydrothorax. ght edema.	4.2	+	2.49	2.51	5.00	0.99	From Sept. 12 to Oct. 30 there was a second period of increasing edema. Weight 31.3 kilos.
t pretibial edema.	4.0	+	2.04	1.51	3.55	1.35	
nt.		+	1.96	3.22	5.18	0.61	From Nov. to Apr. there was steady improvement as far as the edema was concerned.

TABLE

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body we
			gm. per liter		*per cent*	*kg.*
	1922					
No. 3 (J. O'M.).	Oct. 13.	115/70	0.43	25	51	43.6
Male.						
14 yrs.	Nov. 3.	98/50	0.34	27		41.2
Sept., 1922.	Dec. 21.	114/65				40.1
	1923					
	Jan. 23.	112/65	0.27	28		41.8
	Mar. 6.	112/65	0.15	42		44.0
	June 2.	126/65	0.28		45	45.4
	July 21.	138/74	0.28	22		48.0
	1922					
No. 4 (H. M.).	Apr. 10.	104/65	0.405	28	15	18.1
Male.						
7 yrs.	Sept. 25.	95/65	0.397	29	14	17.3
Dec., 1919.	*1923*					
	Apr. 3.	112/74	0.38	31		19.1
	1922					
No. 5 (S. L.).	Apr. 5.	140/70	0.38	22	22	62.0
Male.	Nov. 17.	150/90				62.3
17 yrs.	*1923*					
Oct., 1921.	Mar. 22.	150/115	0.32	14	8	66.1
	Dec. 26.	218/140	0.50	13		65.1
No. 6 (I. O.).	Apr. 13.	170/100	0.42			
Male.						
35 yrs.						
Nov., 1921.						
	1922					
No. 7 (M. H.).	Apr. 28.	140/80	0.43	21	42	49
Female.	July 17.	140/80	0.37	20		47
17 yrs.						
Sept., 1921.						

ntinued.

Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
			Albumin.	Globulin.	Total.	A/G ratio.	
	gm. per 24 hrs.						
rate edema, decreas-	3.6	+++	2.63	2.54	5.17	1.03	Chronic glomerulonephritis.
lema Oct. 23, 1923.	2.0	+++	2.87	2.49	5.36	1.15	Admitted Oct. 8, 1922.
nt.	2.2	++	2.84	1.92	4.76	1.48	Weight 46.5 kilos. Anemia marked; 57 per cent Hb, 3,400,000 r.b.c. in Feb., 1923.
	2.6	+	2.85	1.67	4.52	1.71	Iodide excretion 28 per cent Feb. 20, 1923.
	4.1	+	2.99	1.93	4.92	1.55	Urine urea after urea feeding
	(3.0°/₀₀)	++	2.98	2.32	5.30	1.28	1.3 per cent Mar. 6, 1923.
ess of ankles.	(5.0°/₀₀)	+	2.15	2.04	4.19	1.05	
nt.	Trace.	Absent.	4.06	3.44	7.50	1.18	Chronic glomerulonephritis. No retinitis
	"	Few r. b. c.	4.06	3.46	7.52	1.17	These examinations were made at late and inactive stage of
	0.3	Absent.	4.06	3.76	7.82	1.08	the disease.
ed ascites.	1.3	+	1.84	2.27	4.11	0.81	Chronic glomerulonephritis.
lema or ascites.	3.0	+	2.56	2.46	5.01	1.04	Very great edema. Initial weight 85 kilos.
nt.	3.0	(+)	2.05	3.03	5.08	0.68	Marked isosthenuria.
	4.4	(+)	2.93	2.45	5.38	1.19	
derable anasarca.	5.0	+	2.2	3.2	5.4	0.70	Chronic glomerulonephritis. Outpatient, not further studied.
nt.	0.8	++	4.80	2.25	7.05	2.1	Chronic glomerulonephritis.
	0.7	++	4.0	2.3	6.3	1.74	Admitted with slight edema of face in Nov., 1921.

TABLE

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body we
	1923		*gm. per liter*		*per cent*	*kg.*
No. 8 (M. F.). Female. 25 yrs. Oct., 1922.	Jan. 5.	205/138	0.46	13		65.
	" 12.	174/122	0.80	7.5	8.5	62.
	Feb. 2.	168/110				54.
	" 13.	160/100	0.545	7.6		50.
	Mar. 1.	136/90	0.49	9.2	32	45.
	" 9.	148/100	0.53	7.5		47.
	Apr. 10.	160/100	0.55	7.9	26	47.
	May 23.	170/110	0.64	6.8		48.
	July 13.	144/92	0.63	10.0		50.
	1924 Mar. 5.	115/81	0.97	7.2	9	50
	1922					
No. 9 (W. K.). Male. 11 yrs. Dec., 1921.	Apr. 28.	96/60	0.08	153	59	40
	1923 Jan. 4.	106/55	0.064	141		55
No. 10 (S. Ly.). Male. 15 yrs. Mar., 1922.	" 22.	152/90	0.28	20	5	40
	" 26.	150/100	0.26	22		39
	Mar. 16.	160/98	0.22	21		40
	June 14.	155/100	0.35	16	7	42
	1922					
No. 11 (I. C.). Male. 44 yrs. Apr., 1921.	Apr. 25.	110/70	0.31	28	50	53

ncluded.

Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
			Albumin.	Globulin.	Total.	A/G ratio.	
	gm. per 24 hrs.						
r marked and general dema. Hydrothorax.	2.7	++	2.30	2.30	4.60	1.0	Subacute glomerulonephritis. Chart 3.
	7.6	+	2.16	2.97	5.13	0.73	Albuminuric retinitis. Increasing anemia.
nishing rapidly.	11.0	+	2.13	3.26	5.39	0.65	Relapses on Feb. 3 and Mar. 9 with bloody urine.
edema. No hydro-rax. Feb. 17, 1923.	8.0	++	2.12	3.27	5.39	0.65	Iodide excretion 8 per cent May 9, 1923.
	6.0	+	2.76	3.49	6.25	0.79	Urea concentration test 0.6 per cent May 22, 1923.
	4.0	++	2.46	3.20	5.66	0.77	
currence.	4.0	+	2.17	2.94	5.11	0.74	
	5.0	+	2.30	2.73	5.03	0.84	
nt.	(3.0‰)	Few r. b. c.	2.92	2.95	5.87	0.99	
	(1.5‰)	Very few r. b. c.	3.68	2.89	6.57	1.27	
	Trace.	Few r. b. c.	4.20	2.75	6.95	1.53	Acute glomerulonephritis. Admitted in Feb., 1922. In stage of recovery.
	Absent.	Absent.	4.18	2.08	6.26	2.00	Urea concentration test 1.6 per cent Jan. 4, 1923.
	3.3	(+)	2.32	2.08	4.40	1.11	Subchronic diffuse glomerulonephritis.
	5.4	(+)	2.77	1.75	4.52	1.58	Onset with headaches and vomiting. Ascites and paracentesis in May. No retinal lesions. Slight hyposthenuria.
		(+)	2.54	2.61	5.15	1.01	
	4.5	(+)	2.33	2.54	4.87	0.92	
.	5.0	(+)	3.10	1.76	4.86	1.76	Chronic diffuse glomerulonephritis. Admitted Mar. 9, 1922, with general anasarca. Weight 61.2 kilos. Fundi healthy.

TABLE IV.

Cases of Glomerulonephritis. Vascular Type.

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10\ D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body weight.	Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc. Albumin.	Globulin.	Total.	A/G ratio.	Diagnosis and remarks.
	1923		*gm. per liter*		*per cent*	*kg.*		*gm. per 24 hrs.*						
No. 12 (A. L.). Female. 34 yrs. Dec., 1922.	May 7.	215/140	0.16*	29	10	61.4	Absent.	4.9	Few r. b. c.	3.89	3.18	7.07	1.22	Glomerulonephritis; vascular type. Pyelonephritis, anuria, uremia. Marked albuminuric retinitis. Pericarditis. Died May 26. Autopsy. See text.
	" 24.	170/100	2.64			61.2	"	Anuria.		3.63	3.24	6.87	1.12	
	" 26.	135/45	1.50				"	"		2.99	3.30	6.29	0.91	
	1922													
No. 13 (M. K.). Female. 24 yrs. Oct., 1921.	Oct. 5.	210/120	1.58	4		43.7	Slight pretibial edema.	2.1	Few r. b. c.	3.34	2.57	5.91	1.30	Glomerulonephritis; vascular type. Uremia. Marked albuminuric retinitis. Pericarditis. Died Oct. 11. No autopsy permitted.
	" 11.	130/58	2.95			41.9	Face puffy.	Incontinent.		3.14	2.29	5.43	1.37	
	1923													
No. 14 (J. M.). Male. 27 yrs. 1921.	Oct. 27.	204/140	2.19	2.5		55.8	Slight.	1.2	Very few r. b. c.	2.79	3.22	6.01	0.84	Glomerulonephritis; vascular type. Uremia. Pericarditis. Albuminuric retinitis. Oct. 28. Phlebotomy, 400 cc. Died Oct. 30. See text.
	" 29.	205/140	2.44			55.2		0.5		2.82	2.74	5.56	1.03	
	" 30.	208/136	2.39			55.2			6	2.45	3.19	5.64	0.77	

* Observation dated May 7 confirmed by non-protein nitrogen determination.

teins (Chart 2). In Case 8 the increased concentration of protein accompanying and following the elimination of edema was not maintained, and later fell almost to the initial level without any recurrence of edema (Chart 3). In No. 5 there was a definitely increased protein concentration after absorption of the edema, but there had been an equally marked improvement in his nutrition and general condition. After 2 years and 3 months hypertension was present, presumably associated with hyperplastic sclerosis and a contracting kidney. Nutrition was practically normal. The protein concentration had slightly increased, but only to 5.38 per cent.

In all these cases of low protein concentration in the plasma the loss of protein in the urine was considerable, amounting to from 2 to 5 gm. or more daily. Reversely, when the plasma proteins were within normal limits, the protein loss in the urine was small, 0.8 gm. a day or less.

The reduction of the plasma albumin was greater than that of the plasma globulin, which varied comparatively little, consequently the A/G ratio was found to be below 1.4. The exceptions were Cases 7 and 9 already referred to as having a normal total protein concentration, and Case 11, in which the reduction appears to have affected the globulins chiefly. A/G ratios of 0.8 or 0.6 were not infrequent and occurred with edema as in Cases 1, 2, 6, and 8 and without edema, as in Nos. 1, 2, 5, and 8.

The low A/G ratios occurred in the more severe cases, Cases 2, 5, and 8. These patients presented greater edema than some of the others, but they showed as well a greater increase in the blood urea and blood pressure. In none of them was the passing of the stage of edema and the entry upon that of nitrogen retention associated with a return of the A/G ratio to normal. Cases 4 and 8 had made a very substantial recovery, however, to 1.18 and 1.27 respectively, during the period covered by this study, so that it seems probable that after a more prolonged period the recovery may be complete. The low A/G ratios in these cases were associated with high globulin concentrations.

In one acute case, No. 9, and one chronic case, No. 7, a completely normal condition of the plasma proteins was found in convalescence.

Glomerulonephritis, Vascular Type: (Table IV). Three cases of

TABLE V.

Cases of Nephrosis.

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10\,D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body weight.	Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
										Albumin.	Globulin.	Total.	A/G ratio.	
			gm. per liter		*per cent*	*kg.*		*gm. per 24 hrs.*						
	1922													
No. 15 (L.S.). Male. 30 yrs. Sept., 1923.	Oct. 9.	132/85	0.47	19	44	82.4	Marked edema, hydrothorax and ascites.	3	Very few r. b. c.	1.68	2.48	4.16	0.68	Acute nephrosis. Chart 4.
	Nov. 10.	110/78	0.40	30	55	76.9	Slightly decreased.	7	Absent.	1.52	2.34	3.86	0.65	Recovery, complete.
	" 19.	106/74	0.18			74.3	Elimination of edema commenced on 18th.	5	"	1.51	2.49	4.00	0.61	
	Dec. 4.					62.5	No edema. A little fluid in pleura.	3	"	1.86	2.30	4.16	0.81	
	" 21.	102/60			62	61.5	Absent.	3	"	2.57	2.82	5.39	0.91	
	1923													
	Jan. 9.	124/74				63.5	"	Trace.	"	3.84	2.38	6.22	1.61	
	Feb. 2.	114/65	0.196	48		64.8	"	"	"	3.64	2.24	5.88	1.62	
	Apr. 5.	112/75	0.158	76	59	69.0	"	"	"	3.92	2.84	6.76	1.52	
	Dec. 21.	122/72	0.173	100		73.1	"	Minute trace.	"	4.03	2.70	6.73	1.50	
	1922													
No. 16 (B.S.) Male. 11 yrs. Feb., 1922.	Nov. 6.	95/70	0.07	87	69	34.9	Marked edema of face; slight of shins.	6	"	1.11	3.43	4.54	0.32	Chronic nephrosis. See Chart 5.
	Dec. 12.		0.07	163		33.2	Absent.	2	"	1.67	3.23	4.90	0.52	Urea concentration test 2.4 per cent.
	" 21.	91/68				33.1	"	0.3	"	1.61	3.10	4.71	0.52	Dilution test—slight delay.
	1923													
	Jan. 17.	106	0.09	71		37.4	Slight edema of face and shins.	(2°/₀₀)	"	1.46	3.62	5.08	0.40	Concentration test—normal.

	Mar. 22.	110	0.09*	152		40.6	Hydrothorax, ascites, and slight edema.	(8°/₀₀)	Absent.	0.99	3.36	4.35	0.29	Iodide test 55 per cent.
	Apr. 3.	118	(0.26)†			39.5	Improving slowly.	2	"	0.84	3.20	4.04	0.26	
	" 9.	115				39.4	" "	3	"	0.75	2.84	3.60	0.26	
	May 14.	110/70	0.06	133		34.5	Small ascites.	1	"	1.15	3.28	4.43	0.35	
	" 23.	112/68	(0.25)†			34.1	Absent.	2	"	1.12	3.18	4.32	0.35	
	June 15.	106	(0.19)†			38.6	Small ascites.	3	"	1.02	2.75	3.77	0.37	
	" 26.	110	(0.38)†			37.0	Absent.	1	"	1.17	2.58	3.75	0.45	
	Oct. 8.	100/70	0.07	100	65	38.2	"	(6.5°/₀₀)	"	1.18	3.50	4.68	0.34	Oct., 1923. Dilution and concentration test—normal.
	1924													
	Feb. 13.	118	0.049	88		49.3	General edema, ascites, hydrothorax.	4.5	Very few r. b. c.	0.63	3.11	3.74	0.20	
	" 27.	108/75	0.24*	100	70	43.6	Steady slow improvement.	1.2	Absent.	1.04	3.33	4.37	0.31	
	Mar. 10.	106	0.20*†			37.0	No edema or ascites.	0.6	"			4.15		
	1923													
No. 17 (H.J.S.). Male. 32 yrs. Jan., 1923.	Jan. 29.	130/75	0.11	40	33	62.3	Marked general edema.	2	"	2.85	2.82	5.67	1.01	Acute nephrosis following influenza. Weight fell from 64 to 57 with disappearance of edema. Recovery rapid and complete.
	1922													
No. 18 (F. R.). Female. 20 yrs. Oct., 1921.	Mar. 23.	98/65				56.3	Slight edema.	1	Very few r. b. c.	3.2	3.5	6.7	0.91	Acute nephrosis; stage of recovery. Admitted in Nov. with moderate edema which increased greatly in Dec. and then subsided. Maximum weight 66.9. Complete recovery.
	Apr. 27.	100/70	0.155	69	64	57.8	Absent.	Tracc.	Absent.	3.87	3.47	7.34	1.11	
	1923													
	June 11.	126/75	0.065	81		60.7	"	"	"	4.82	2.94	7.76	1.64	

* Following urea feeding therapy.

† Plasma non-protein nitrogen.

TABLE V—*Concluded.*

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10\,D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body weight.	Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
										Albumin.	Globulin.	Total.	A/G ratio.	
	1923		*gm. per liter*		*per cent*	*kg.*		*gm. per 24 hrs.*						
No. 19 (G. F.). Male. 51 yrs. 1922.	June 13.	118/78	0.076	81	63	83.0	Slight.	7.0	Moderate. No r.b.c.	2.19	2.33	4.52	0.94	Chronic nephrosis. Urine urea after urea feeding 2.2 per cent.
	July 16.	135/85	0.20	30		80.8	Very slight.	5.1	" "	1.98	2.33	4.31	0.85	Dilution test—normal.
	Sept. 12.	135/80				81.6	Considerable.	6.0	Absent.	1.48	2.79	4.27	0.53	Concentration test—normal.
	Nov. 27.	135/80	0.067			81.5	Massive.	5.1	Few r. b. c.	1.33	2.49	3.82	0.53	Dec. 15, 1923. Blood creatinine 1.7 mg. per 100 cc.; blood uric acid 2.9 mg. per 100 cc.
	Dec. 15.	130/80	0.145‡			74.3	Very slight.	(3.0°/$_{oo}$)	Very few.	1.28	3.00	4.28	0.38	
	1924 Jan. 30.	119	0.195‡	48	60	74.4	Slight pitting of ankles at night.	(0.2°/$_{oo}$)	" "	1.78	2.42	4.20	0.74	

‡ During urea feeding therapy.

this condition were studied during the final stage of their illness, and in two of them a postmortem examination was made. In all of them the total concentration of proteins in the plasma remained normal until a few days before death, but in Cases 13 and 14 a terminal fall to about 5.5 per cent did occur before death. In Case 12 the figures show a rapid fall in the concentration of albumin, but the globulin concentration increased to a small extent; the total therefore fell considerably although it remained within the normal limits till the end. Case 14 too showed a definite increase in the globulin concentration. As a consequence the A/G ratio was reduced.

The figures for the protein in the urine probably do not adequately represent the loss of protein by this route. Rose Bradford (27) observed that, although the concentration of protein in the urine might be moderate or small in these cases, yet on account of the polyuria the loss of protein was greater than in other varieties of Bright's disease. In our cases the polyuria was failing, and probably the loss of protein observed was not so large as it had been before this failure occurred.

Nephrosis: Five cases (Table V). Two of these were severe and typical and were studied in detail. In the four patients (Cases 15, 16, 17, and 19) observed during the stage of edema and marked proteinuria, there was reduction of the total protein concentration in the plasma, which was slight in the mild acute case, No. 17, but was well marked in the three severe cases. In No. 15 the protein concentration was 4 per cent when the edema was greatest, and it remained unchanged until after the striking elimination of edema which ended about December 6. 2 weeks later the concentration had increased to 5 per cent, and after the fall of proteinuria to a trace, it rapidly rose to normal (Chart 4). The observations on No. 18 were obtained in the stage of advanced recovery and showed the same return to normal. In Case 16 the initial elimination of a small amount of edema was observed; after a period of well-being this was followed by one of slowly increasing fluid accumulation. The protein concentration rose from 4.5 to 5.0 per cent. With the sudden formation of pleural and peritoneal effusions in March, the proteins fell to 4.35 per cent. This fall continued to a minimum of 3.6 per cent in spite of slight clinical improvement. Under thyroid therapy the patient began to im-

TABLE VI.

Cases of Nephrosclerosis.

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10\,D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body weight.	Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis and remarks.
										Albumin.	Globulin.	Total.	A/G ratio.	
	1922		*gm. per liter*		*per cent*	*kg.*		*gm. per 24 hrs.*						
No. 20 (E. R.). Male. 43 yrs. Since 1919.	Apr. 7.	228/125	0.23	36	43	71	Absent.	0.7	Few r. b. c.	4.1	2.9	7.0	1.41	Nephrosclerosis (advanced). Retinal hemorrhages; "silver wire" arteries. Marked polyuria and isosthenuria. Died outside hospital in July, 1922. No autopsy.
No. 21 (S. J.). Female. 57 yrs. Since 1920.	Nov. 11.	180/110	0.120			66	Slight edema of ankles.	Absent.	Absent.	4.59	2.47	7.06	1.86	Nephrosclerosis. Fundi natural. Temporary aphasia. Paresthesia. Moderate myocardial insufficiency.
No. 22 (H. L.). Female. 29 yrs. Mar., 1922.	" 14. *1923*	215/130	0.154	47	61	51	Absent.	Trace.	"	4.54	2.87	7.41	1.58	Nephrosclerosis. Fundi natural. Renal function normal. Urine urea after urea feeding 2.8 per cent, Dec. 22, 1922.
	May 2.		0.085	87	70		"	"	"					

No. 23 (H. Ln.). Male. 57 yrs. Mar., 1922.	Mar. 2.	220/130	0.21			77.5	Edema of legs, back, and lungs.	1.0	Absent.	4.17	3.35	7.52	1.25	Nephrosclerosis. Myocardial insufficiency. Successful digitalis therapy.
	" 13.	188/102	0.15	23	29	70.2	Slight edema of legs.	0.2	"	3.44	2.90	6.34	1.19	
No. 24 (C. T.). Female. 34 yrs. 1920.	Nov. 22.	175/105	0.15	32		76	Absent.	0.98	"	4.27	2.94	7.21	1.45	Nephrosclerosis. Fundi natural. Renal function normal. Urine maximum. Specific gravity 1,032; urea 2.7 per cent.

TABLE VII.

Cases of Functional Proteinuria.

Case No. Age. Date of initial symptom.	Date of observation.	Blood pressure.	Blood urea nitrogen.	Urea index. $\frac{10\,D}{B\sqrt{VW}}$	Phthalein 2 hrs.	Body weight.	Edema. Ascites. Hydrothorax.	Protein in urine.	Hematuria.	Plasma proteins in gm. per 100 cc.				Diagnosis. Remarks.
										Albumin.	Globulin.	Total.	A/G ratio.	
	1922		*gm. per liter*		*per cent*	*kg.*		*gm. per 24 hrs.*						
No. 25 (H. B.). Male. 20 yrs. Feb., 1921.	July 10.	100/70	0.106	84	61	50.4	Absent.	Trace.	Absent.	4.06	3.99	8.05	1.02	Proteinuria; chronic tonsilitis. No retinitis or organic cardiovascular changes. Renal function tests—normal response. Proteinuria sometimes 0.5 ‰ with an occasional r. b. c.
No. 26 (G. S.). Female. 19 yrs. Nov., 1920.	Nov. 10.	118/80	0.08	106	65	43.0	"	Occasional trace.	"	4.00	2.51	6.51	1.60	Proteinuria; cardiovascular asthenia. A very definite history of a high grade proteinuria was obtained from her physician. No organic disease. Renal function normal.

prove rapidly and the effusions were quickly absorbed; the protein concentration rose to just over 4 per cent (May 14 and 23). In an attempt to increase this concentration, a diet containing 100 gm. protein, 30 gm. fat, and 300 gm. carbohydrate was given from May 26 to June 4, during which period he gained 1.5 kilos in weight. The protein was then increased to 140 gm. daily, 3.9 gm. per kilo of body weight, and this diet was continued until June 15. During this period he was not so well as during the period ending May 26, when he was getting 80 gm. of protein daily. He gained in weight and his abdomen increased in size and contained a little free fluid. Associated with this there was an actual fall in the plasma protein concentration to 3.77 per cent. With a return to diet of 80 gm. protein daily the patient improved generally and the signs of ascites disappeared. The protein concentration, however, 10 days later had not increased. The proteinuria in this case was smallest at the times of clinical improvement.

The daily loss of protein in the urine of these cases of nephrosis with low protein concentration was roughly the same as that observed in similar cases of glomerulonephritis. Periods of clinical improvement coincided with periods of low proteinuria.

The fall in the A/G ratio was marked in Cases 15, 16, and 19, particularly in the last two. In Case 16 the decrease of albumin accounted for the entire fall in the plasma proteins, and was carried to such a degree that figures of 0.99, 0.75, and 0.63 per cent were found during the period of great serous effusions, these corresponding to A/G ratios of 0.29, 0.26, and 0.20. With absorption of the effusions the A/G ratio remained at 0.35. On no occasion was a subnormal figure for the globulins obtained. In Case 15 we found similar but less extreme changes; the variation in the albumin was 2.41 per cent and in the globulin 0.6 per cent. In Case 19 there was in the first instance a considerable fall in the albumin and a small fall in the globulin, and as the disease progressed the albumin concentration steadily diminished while the globulin increased. The A/G ratio fell from 0.94 to 0.38. A considerable period elapsed before this characteristic picture was developed.

In Cases 15 and 18 the process of recovery in nephrosis was well demonstrated.

Nephrosclerosis: Five cases (Table VI). In none of these was there any evidence of a true nephritis, and in all except No. 20 renal function was good. No. 21 had slight cardiac insufficiency and in No. 23 it was conspicuous. The total protein concentration was normal in all of them, and the A/G ratio was normal with one exception. Proteinuria was slight in all these cases.

Functional Proteinuria:—Two cases (Table VII). Both these patients presented a feeble cardiovascular system with sluggish peripheral circulation, and an unstable nervous system. In Case 25 the proteinuria was discovered at examination following a paroxysm of tachycardia. The protein was constantly present and there was about ten times as much albumin as globulin, a result differing from those obtained by Mackenzie Wallis (28) in similar cases. In Case 26 traces of protein were found infrequently, but a reliable history of a higher grade of proteinuria was obtained from her physician. Neither patient showed any sign of renal insufficiency. In both the plasma protein concentration was normal, but in Case 25 there was an increase in globulin possibly to be accounted for by the chronic sepsis in his tonsils (Rowe (10)).

DISCUSSION OF RESULTS.

The observations which are reported here agree with the conclusions of most previous workers that in chronic nephritis with edema, that is in nephrosis and the nephrotic type of glomerulonephritis, there is a great fall in the concentration of the proteins in the plasma, and that this fall occurs more especially in the albumins; consequently the A/G ratio falls below the normal minimum of 1.4. But it is also apparent from these figures that although this change occurred in all cases with edema, and although the lowest figures for the plasma protein concentration were obtained when some edema was present, yet the greatest decrease in concentration did not correspond with the greatest edema, and a very considerable decrease was compatible with absence of recognizable edema.

The hypothesis has been repeatedly advanced that the cause of edema in nephritis is the decrease in the osmotic pressure of the plasma which in turn is due to the small amount of protein in the plasma. If this were true it is probable that the lowest protein concentration in

the plasma would be found during the stages of increasing and of fully developed edema, and that the first sign of a change for the better would be a rise in the osmotic pressure of the plasma consequent upon an increase in the plasma proteins. In the results reported here no such close relationship appeared. Case 2 showed no connection between variations in edema and variations in plasma protein concentration; in No. 3 a lower concentration was present in the later stages after the edema had gone; No. 15 showed a trivial increase during his great diuresis and elimination of edema, and a rapid return to the normal level at a later date; in No. 16 the concentration was 3.6 per cent during a period of improvement (April 9), and 4.35 per cent at the height of his edema (March 22); in No. 19 the total protein concentration varied little, whether edema was present or absent. The conclusion, therefore, is that there is no direct relationship of cause and effect between the low protein concentration in the plasma and the presence and degree of edema. That the passage of protein into the tissue spaces in edema is the prime factor in producing the decreased concentration of proteins appears improbable. In nephritis the concentration of protein in the edema fluid is small, 0.1 to 0.17 per cent according to Epstein (29), and the proportion of globulin is high, 50 to 80 per cent; whereas the loss of protein from the plasma is large, as we show in a subsequent paper, and the protein most deficient is albumin.

The reduction of the A/G ratio was of similar degree in glomerulonephritis of the nephrotic type and in acute nephrosis; but in the two cases of chronic nephrosis, Nos. 16 and 19, this reduction was much more pronounced. This disturbance of the A/G ratio depended in some instances upon a relatively greater decrease of albumin than of globulin, but in others an absolute increase in globulin concentration occurred with or without a decrease in albumin. In Cases 4, 6, 8, 12, 14, 16, 18, 23, and 25 the globulin concentration was definitely higher than in normal persons; an absolute increase in globulin concentration was present. Erben (30), 1903, found increases of globulin up to 7 per cent with decrease of albumin to 0.2 per cent, but his results were probably faulty, for no other observer has found figures comparable to these. It has been found that similar increases in globulin may occur in pneumonia, syphilis, and other infections and

intoxications (Rowe (10), Hurwitz and Whipple (31)) as well as in disturbances of metabolism such as diabetes mellitus (Epstein (14)). Evidence is steadily accumulating to show that nephritis is a disease not merely of the kidneys, but that it includes widespread changes in remote tissues and fundamental if still obscure changes in metabolism. The increase in globulin may well be a phase of this metabolic derangement. Possibly the formation of plasma globulins is the primitive form of plasma protein production, and the organism in disease tends to return to this primitive form; the figures Halliburton (13) reported for cold blooded animals give a little support to this speculation.

It is doubtless true that the loss of protein in the urine is an important factor in reducing the concentration of the plasma proteins and in disturbing the A/G ratio. In the great majority of the patients with proteinuria exceeding 1 gm. a day there was reduction of the total concentration and the ratio. Reversely, in no instance did the concentration remain persistently abnormal in patients losing protein in smaller amounts. The A/G ratio, however, was low in some cases when the urine contained merely a trace of protein and when the total concentration of protein in the plasma was unchanged (Cases 4, 18, 23, 25). Similarly when the proteinuria diminished in Case 18 and the plasma protein concentration returned to normal the A/G ratio did not do so for a considerable period. No systematic study was made of the A/G ratio in the urine of these patients, but in the few determinations made the amount of albumin was many times greater than that of globulin, agreeing with the findings of Hoffmann (32) and Wallis (28); the greater loss of albumin in the urine is presumably an important factor in the greater decrease of albumin in the blood. On the other hand loss of protein in the urine cannot explain the absolute increase in globulin concentration referred to above, and closer examination of the figures shows that this increase in globulin was the chief reason for the persistence of the low A/G ratio in Cases 4, 18, 23, and 25. In Case 12 we found a combination of high proteinuria with a normal plasma protein concentration; in the last days of this patient's life anuria was present, and this was associated with a rapid fall in the concentration of the plasma albumin and a slight rise in that of the globulin. These observations cannot be explained on the basis of

proteinuria nor by the assumption that a hydremic plethora had occurred. In Case 8 the plasma proteins reached a concentration of 6.2 per cent in spite of a high grade of proteinuria. Loss of protein in the urine, therefore, is insufficient to explain by itself the changes we have observed.

A correlation of tubular degeneration with edema and low plasma protein concentration is unsatisfactory. Fatty degeneration of the tubules is the conspicuous feature of the histological picture in nephrosis and it is well marked in the nephrotic type of glomerulonephritis. It may, however, be equally apparent in the vascular type of glomerulonephritis; in Cases 12 and 14 there were advanced fatty changes in the tubules.

The diet is probably a secondary factor of some importance in the fall of the total protein concentration and of the A/G ratio; the low protein diet so frequently given to all cases of proteinuria may well have a deterrent effect on the adequate production of plasma proteins, especially when associated with a considerable loss of proteins in the urine. Kerr, Hurwitz, and Whipple (33) observed the regeneration of the plasma proteins in dogs after repeated washings of the blood had reduced these proteins to a very low level. They found that globulin was produced more rapidly than albumin, that regeneration was faster when food was given than when the animals fasted, and that regeneration was faster when meat was given than when the diet was restricted to bread and milk. These observations appear of considerable importance in the treatment of nephritis.

An explanation commonly advanced to account for these decreases in the concentration of the plasma proteins is that a condition of hydremic plethora exists in these cases, and that the fall in concentration is an index of a latent or internal edema, an edema of the blood itself. In a succeeding paper evidence is presented which shows that a condition of hydremic plethora does not occur even in cases of great edema and that there is a definite decrease in the absolute amount of plasma proteins in the body.

It appears probable, therefore, that two factors are responsible for the changes in the plasma protein concentration in nephritis. They are, first and more important, a disturbance in the mechanism of their production consequent upon the nature of the disease, the presence of

infection or toxemia, and dietary vagaries; and second, the loss in the urine of large amounts of protein, particularly albumin. Although low protein concentrations and edema so frequently occur together, their relationship is not a simple one of cause and effect; they and the increase in blood lipoids are probably the expression of an underlying metabolic disorder of a fundamental nature.

In nephrosclerosis and in two cases of "functional proteinuria" no changes in the plasma protein concentration or the A/G ratio were found. The position in regard to "functional proteinuria" is not clear, for both our patients had but small amounts of protein in the urine.

SUMMARY.

In nephrosclerosis (Volhard and Fahr classification of nephritis) there was no decrease in the plasma proteins. With heart failure the ratio of albumin to globulin fell from the normal 1.7 ± 0.3 to about 1.

There is a sharp difference between the two types of glomerulonephritis in the effect on plasma proteins. In the vascular-interstitial type the effect was the same as in nephrosclerosis. There was no decrease in the plasma proteins until shortly before death.

In the glomerulotubular or nephrotic type, active or recently active, the total plasma proteins were less than 5 gm. per 100 cc. This decrease from the normal 5.5 to 7.5 occurred whether edema was present or absent. The decrease affected chiefly the albumin, the globulin being usually diminished but little, and sometimes slightly increased. Consequently the ratio of albumin to globulin was reduced to less than 1, and occasionally to 0.6.

In nephrosis similar changes were found in the total protein concentration but in severe cases the decrease in albumin was greater than in nephrotic glomerulonephritis while the globulin was either very slightly reduced or was increased. The albumin : globulin ratio was therefore lower than in nephrotic glomerulonephritis, ranging down to 0.26.

With the disappearance of edema there was usually an increase in the plasma protein concentration, but this was not invariable, and concentrations of 4.5 per cent or less were compatible with the persistent absence of edema. The ratio of albumin to globulin showed an irregular tendency to rise. Whenever the total concentration was

less than 4 per cent there was some edema present, but it was sometimes slight in amount.

With recovery in acute cases, and remission with decrease of proteinuria in chronic cases, normal concentrations were regained. The albumin : globulin ratio remained low in some instances for a longer period on account of an absolute increase in globulin concentration.

In "functional proteinuria" no change in plasma proteins was found.

Low plasma protein concentrations were always associated with considerable losses of protein in the urine, but these losses did not provide an explanation for all our observations. A disturbance in the production of the plasma proteins appears probable.

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CHANGES IN THE VOLUME OF PLASMA AND ABSOLUTE AMOUNT OF PLASMA PROTEINS IN NEPHRITIS.

By G. C. LINDER, M.D., C. LUNDSGAARD, M.D., D. D. VAN SLYKE, Ph.D., and E. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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In a preceding paper (1) it was shown that a considerable fall occurred in the concentration of protein in the plasma of persons suffering from nephrosis or from glomerulonephritis of the nephrotic type. In discussing this abnormality of the plasma proteins the question arose whether it was due in any degree to a process of dilution; whether a plasma previously normal in volume and protein concentration became greater in volume and poorer in proteins by the retention of extraneous water in the blood. This hypothetical condition has been named hydremic plethora, and it was tacitly assumed to be present in some stages of nephritis by most of the early workers who found a reduced concentration of the plasma proteins. In the absence of any definite means for distinguishing clinically between hydremic plethora and simple hydremia, that is between a dilute state of the blood with and without increase in total volume, these terms were vaguely used, and these early opinions were of little scientific value (2). An extension of this hypothesis connected the assumed changes of blood volume with the production of edema. Edema was thus believed first to involve the blood itself and then to implicate the tissues by reducing the osmotic pressure of the plasma. Similarly the anemia of nephritis was explained on the same basis as a dilution anemia. Brown and Roth (3), however, in a recent study concluded that the anemia of chronic nephritis is not due to dilution.

Whether low concentration of the plasma proteins also occurs independently of plasma volume change, or affords an indication of increased volume, remained undetermined. The data in the present paper appear to answer the question decisively. We have observed a series of nephritic patients with low plasma protein concentration,

with all degrees of edema, and have not found an abnormally large plasma volume per kilo of body weight, as determined by the vital red method of Keith, Rowntree, and Geraghty.

A number of observations on the blood and plasma volumes in nephritis have been published but none have come to our attention in which the plasma protein concentrations were simultaneously determined. Plesch (4), 1909, found an increased volume of blood in three cases of nephritis without edema and a marked decrease in one case with edema. He concluded that the increase was the decisive factor in the production of hypertension and that edema was a compensatory mechanism by which the effects of this increase on the blood pressure were mitigated. Keith, Rowntree, and Geraghty (5) reported nineteen measurements by the vital red method on cases of chronic nephritis and hypertension. In one case with considerable edema the plasma volume increased from 3,900 to 4,050, while the patient lost 11 kilos of edema. In the other cases they found an average of 42.8 cc. of plasma and 75 cc. of blood per kilo body weight, their normal figures being 42 to 56 and 78 to 97 respectively. There was, therefore, no increase. Plesch (6), 1922, using the carbon monoxide method found increases of blood volume in all cases of nephritis and arteriosclerosis. In two edematous patients the blood volume and hemoglobin increased as the edema decreased. Bock (7), 1921, reported observations by the dye method on three cases of nephritis, in two of which the plasma amounted to 4.8 and 6.0 per cent of the body weight. The third patient had great edema, the weight falling from 54.5 to 41 kilos in 1 month. The plasma per kilo observed body weight remained constant (4.0, 4.1, and 4.1), but the figures show a marked fall in absolute plasma volume (2,200, 1,900, and 1,690 cc.). Seyderhelm and Lampe (8) determined the rate of disappearance of trypan red from the circulation in normal people and in patients with nephritis. They found that in cases of great edema 50 per cent of the dye left the circulation in half an hour without any appreciable amount appearing in the urine during that time. In normal persons only 5 to 10 per cent disappeared in that time. They think that in such cases the dye must pass into the tissues. They failed to find increases in blood volume in nephritis (9) even when accompanied by anemia or edema, but state: "Eher war sogar die Gesamtblutmenge oft in hochgradigsten Status oedematosus mit sogenannter Hydrämie ausgesprochen vermindert."

Methods.

The methods useful for the determination of the absolute volumes of the plasma and blood in clinical practice are two: first, the carbon monoxide inhalation method, introduced by Gréhant and Quinquaud (10), applied to man by Haldane and Lorrain Smith (11), and improved by Van Slyke and Salvesen (12, 13), and second, the method

of injecting a non-diffusible dye, such as vital red, which was first employed by Keith, Rowntree, and Geraghty (5). These methods have been tested by a number of investigators (14–18), and their limitations have been thoroughly discussed, particularly by Whipple and his associates (14, 15) and by Lamson and Rosenthal (16). The conclusion appears to be that while neither method can be depended upon to give absolute results, either one may be used to observe variations in the blood volume of a given individual.

In this investigation the original vital red method of Keith, Rowntree, and Geraghty was used (5). The measurements were made about 2 hours after a breakfast which was arranged to contain as little fat as possible; by this precaution cloudiness of the plasma was greatly reduced. In consequence of the probable inaccuracy of the results depending upon the hematocrit we did not attach so much importance to the figures for the blood volume as to those for the plasma volume. Oxalate was employed as anticoagulant. In view of Lamson's criticism we made repeated observations on our patients when that was possible, but we found that the comparison of the findings from single determinations in other cases was not valueless. Keith, Rowntree, and Geraghty (5) found the plasma to vary in health between 43 and 54 cc. per kilo of body weight, and Bock (7) found it to be from 4.5 to 5.7 per cent of the body weight. For purposes of comparison we assumed a standard body weight for each patient, which was the weight reached when the patient became free from edema. This standard body weight was used in all the calculations for that individual. In some instances this weight was derived from the age, height, and weight tables because the patient did not become edema-free. The weight of a patient with nephritis when free from edema is liable to be less than it should be, as cachexia is a feature of the disease. No attempt was made to correct for this.

The patients used were those described in the preceding paper on the plasma protein concentration and the clinical details will not be repeated here. The same case numbers are used and the present results are recorded in the table. Charts illustrating some of the plasma volume findings will be found in the preceding paper.

Results of Plasma Volume Measurements.—Glomerulonephritis and Nephrosis: In four patients (Cases 1, 8, 15, and 16) observations

TABLE I.

Case. Diagnosis.	Date.	Actual weight.	Estimated weight. Edema-free.	Edema, etc.	Plasma volume.		Plasma protein in gm. per kilo body weight.			Red blood corpuscles in millions per c.mm.	Cell volume.	Blood volume.		Red blood corpuscles per kilo estimated body weight.
					Cc.	Percentage of body weight.	Albumin.	Globulin.	Total protein.			Cc.	Percentage of body weight.	
	1922	*kg.*	*kg.*								*per cent*			
(Normal.)						(5)	(2.05)	(1.3)	(3.35)	(5.0)	(43.0)		(8.8)	4.2×10^{11}
No. 1 (J. D.).	May 20	50.4	38	+++	2,060	5.42	0.86	1.14	2.00	4.15	31.0	2,980	8.24	3.25
Subchronic glomerulonephritis, nephrotic type.	June 7	39.0		+	2,060	5.42	1.25	0.98	2.23	—	32.5	3,120	8.62	—
	July 10	38.8		(+)	2,080	5.48	1.06	1.16	2.22	3.49	24.9	2,770	7.65	2.55
	Nov. 2	36.8		—	2 080	5.48	1.22	1.25	2.47	3.38	22.8	2,690	7.43	2.39
	1923													
	Jan. 5	38.2		—	1,960	5.32	1.37	1.03	2.40	2.95	19.8	2,440	6.74	1.89
	Mar. 9	41.6		—	2,270	5.98	1.36	1.39	2.75	2.48	17.6	2,750	7.60	1.80
No. 8. (M. P.).	Jan. 5	65.8	48	++++	2,470	5.30	1.18	1.18	2.36	3.52	27.7	3,420	7.48	2.51
Subacute glomerulonephritis, nephrotic type.	Feb. 2	54.8		++	2,790	5.99	1.24	1.89	3.13	2.60	18.3	3,420	7.48	1.85
	Mar. 9	47.9		—	2,650	5.69	1.36	1.77	3.13	3.08	23.7	3,475	7.60	2.23
	1922													
No. 15 (L. S.).	Oct. 9	82.4	64	++++	2,720	4.38	0.71	1.05	1.76	—	39.8	4,520	7.42	—
Acute nephrosis.	Nov. 19	74.3		+++	2,795	4.50	0.66	1.09	1.75	4.18	36.6	4,410	7.24	2.88
	1923													
	Feb. 2	64.8		—	2,960	4.76	1.68	1.04	2.72	4.82	38.8	4,840	7.94	3.65
	1922													
No. 16 (B. S.).	Nov. 6	34.9	35	+	1,980	5.83	0.63	1.94	2.57	5.08	38.5	3,210	9.64	4.66
Chronic nephrosis.	Dec. 21	33.1		—	1,520	4.48	0.70	1.35	2.05	4.75	37.7	2,440	7.32	3.31
	1923													
	Mar. 22	40.6		Ascites, etc., ++	1,650	4.86	0.47	1.58	2.05	4.72	37.0	2,620	7.86	3.53

	Apr. 9	39.4		Ascites, etc., +	1,520	4.48	0.33	1.24	1.57	5.50	42.0	2,620	7.86	4.12
	June 26	37.0		Absent.	1,620	4.77	0.54	1.19	1.73	4.65	34.9	2,490	7.5	3.31
	1922													
No. 3 (J. O'M.). Chronic glomerulonephritis, nephrotic type.	Nov. 3	41.2	42	–	2,350	5.76	1.61	1.39	3.00	3.82	27.6	3,250	7.7	2.95
	1923													
	Jan. 23	41.8		–	2,440	5.98	1.66	0.97	2.63	3.36	26.8	3,330	7.9	2.66
	Mar. 6	44.0		–	2,520	6.18	1.79	1.16	2.95	2.63	20.1	3,160	7.5	1.98
	1923													
No. 5 (S. L.). Chronic glomerulonephritis, nephrotic type.	Apr. 5	62.0	54	Ascites.	2,330	4.4	0.81	0.98	1.79	4.2	31.7	3,415	6.6	2.65
No. 9 (W. K.). Acute glomerulonephritis.*	Mar. 28	42.7	42.7	–	1,765	4.25	1.72	1.13	2.85	4.7	40.0	2,940	7.2	3.24
No. 11 (I. C.). Chronic glomerulonephritis, nephrotic type.	May 6	52.7	48	+	2,580	5.54	–	–	–	–	31.0	3,740	8.2	–
No. 20 (E. R.). Nephrosclerosis.	Apr. 7	71.0	71	–	3,330	4.84	1.92	1.35	3.27	–	38.0	5,380	7.9	–
No. 22 (H. L.). Nephrosclerosis.	Nov. 14	51.0	51	–	2,310	4.67	2.06	1.30	3.36	4.54	42.0	3,970	8.2	3.53
No. 13 (M. K.). Glomerulonephritis, vascular-interstitial type.	Oct. 9	42.3	42	(+)	2,880	7.08	2.15	1.57	3.72	1.6	13.5	3,330	8.3	1.27
No. 25 (H. B.). Functional proteinuria.	July 10	50.4	50.4	–	2,270	4.65	1.83	1.80	3.63	–	41.0	3,840	7.6	–

*Convalescent.

were made on the plasma volume before, during, and after the disappearance of extensive general edema. In the first case the plasma volume remained constant while the weight fell from 50.4 to 39 kilos in 17 days (Case 1, subchronic glomerulonephritis, Chart 1 of the preceding paper). In Case 8 (subacute glomerulonephritis, Chart 3), there were at the time of the initial observation general anasarca, pleural effusions, and ascites; with the steady improvement of the clinical condition, there was a definite small increase in the volume of plasma. In the third (Case 15, acute nephrosis, Chart 4), the observations were made before, during, and after a most striking diuresis and elimination of edema, and as in the preceding case, there was an increase in the volume of plasma as the edema disappeared. In the fourth case (Case 16, chronic nephrosis, Chart 5), the results were not so uniform. The initial figure which was obtained when the patient was slightly edematous was probably too high, but as no error in technique could be discovered the observation must stand. No adequate explanation was found to account for this high plasma volume and correspondingly high blood volume and number of red cells per kilo of body weight. If we neglect this observation, there remain four measurements, one before the development of considerable ascites, pleural effusion, and slight edema, one at their fullest development, one in the early stage of recovery, and finally one when all the effusions had disappeared and the patient had returned to a better state of nutrition. The plasma volume was constant at all these times within the limits of error of the method.

Consideration of these four cases shows that the volume of plasma was either uninfluenced by the presence of edema or else that it was actually diminished when the anasarca was extreme. In Cases 8 and 15 the increase which occurred as the edema disappeared was moderate but quite definite; a similar increase was found by Keith, Rowntree, and Geraghty in their case, but Bock as has been already noted found a decrease. The effect of the increased permeability of the capillaries for the dye in cases of great edema as observed by Seyderhelm and Lampe would be to introduce an error which would operate in the direction of increasing the observed plasma volume in the stage of edema. This consideration adds weight to the increases observed in our cases when the edema was absorbed.

Considering all the patients in this group we found that the plasma varied between 4 and 6 per cent of the body weight, but usually between 4.5 and 5.5 per cent. The table shows that in all those cases in which the plasma was above 5 per cent of the body weight, there was a considerable degree of secondary anemia with the red cell count reduced to less than 4.1 million per c.mm.; and, reversely, that in every case in which the plasma was less than 5 per cent anemia of this degree was lacking. To this statement there is one exception, namely the first observation on Case 16, the validity of which we doubt. The secondary anemia of chronic nephritis is frequently but a part of a general cachexia; the high percental weight of the plasma in nephritis with anemia is probably to be accounted for in part by this fact, as these patients are very likely to be under their proper normal weights when they are finally free from edema.

Only one observation was made in glomerulonephritis of the vascular type. This patient, Case 13, presented a very great increase in plasma, and with this an absolute increase in plasma proteins. This suggests that either there was an error in the determination or else the increase in plasma was of some standing and not merely a terminal event. Lorrain Smith (11), Plesch (4), and Keith, Rowntree, and Geraghty (5) report increases in blood volume in severe anemia. As this patient presented such an anemia, the red cells being reduced to 1.6 million per c.mm., it is probable that anemia was the cause of this increase in plasma volume. The volume of the whole blood in this case was not increased.

Nephrosclerosis: One determination was made on each of two cases of nephrosclerosis. In Case 22 the disease was in an early stage and in Case 20 it was well advanced; neither was complicated by heart failure. Both had normal amounts of plasma and of blood for their body weight.

Functional Proteinuria: A single observation fell within normal limits.

Results of the Measurement of Whole Blood and Corpuscle Content.—In consequence of the slight increase in plasma when anemia was well marked, the figures for the whole blood expressed per kilo of body weight were more constant than the corresponding figures for the plasma. They varied between 7 and 8.3 per cent, and were

therefore lower than the figures 8.2 to 10 per cent, given as normal by Keith, Rowntree, and Geraghty (5).

The figures for the red blood corpuscles calculated per kilo body weight lend support to the opinion that dilution of the blood is not the cause of the anemia in nephritis.

Results of the Determinations of the Total Plasma Proteins.—Glomerulonephritis and Nephrosis: If we accept the plasma in healthy adults to be 5.0 per cent of the body weight (4.3 to 5.4—Keith, Rowntree, and Geraghty (5); 4.5 to 5.7—Bock (7)), then, calculating from the figures for the normal plasma protein concentration given in our previous paper, we find that the figures for the absolute amount of albumin, globulin, and total protein are about 2.0, 1.3, and 3.3 gm. per kilo body weight respectively. From the table it is apparent that the low concentration of protein in the plasma was associated with a fall in the absolute amount of these proteins in the body. As for the plasma itself, the plasma proteins were calculated for a standard body weight for each individual, edema-free. In those patients with severe anemia there was a small increase in plasma per kilo, but this seldom exceeded the normal limits and was entirely insufficient to account for the low concentration of proteins on the basis of dilution, or hydremic plethora, as the absolute protein figures plainly demonstrated.

The total globulin varied very much less than the total albumin, a fact which tells very strongly against the explanation of the fall in protein concentration on the hypothesis of simple dilution. The albumin when abnormal was always decreased, never increased; a moderate fall appeared in all the cases of glomerulonephritis, and in most cases it was well marked, but extreme reductions to less than 0.8 gm. per kilo were only found in the two cases of active nephrosis (Nos. 15 and 16). The globulin usually showed a moderate decrease, the lowest figures, 0.98 gm. per kilo, being found in glomerulonephritis (Cases 1 and 5); but in some cases of glomerulonephritis and nephrosis it was increased to as much as 1.89 gm. per kilo (Cases 8 and 16).

Nephrosclerosis and Functional Proteinuria: In nephrosclerosis normal figures were found. In the patient with functional proteinuria there was an increase of globulin and of total protein with a

small decrease in albumin. Possibly the increase in globulin was due to septic absorbtion for he had hypertrophied and infected tonsils which required removal.

CONCLUSIONS.

1. We have not observed gross increases in plasma volume in glomerulonephritis, nephrosis, or nephrosclerosis, even when the concentration of plasma proteins was much below normal. Our results indicate the probability that "hydremic plethora" does not occur.

2. The low protein concentration frequently observed in the plasma in nephritis is not due to increased plasma volume but to a decrease of the total amount of plasma protein in the body.

3. Changes in plasma volume showed no constant relationship to changes in edema.

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FAT METABOLISM IN NEPHRITIS.

By A. HILLER, G. C. LINDER, M.D., C. LUNDSGAARD, M.D., AND D. D. VAN SLYKE, PH.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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It has been known for some time that in some cases of nephritis there is an increase in the blood lipoids, sometimes to the extent of 100 per cent or more above the normal. A review of the literature to 1917 has been given by Bloor (1). Later investigations confirmed these results. Epstein and Rothschild (2) thought the high lipoids in nephritis represented a condition of fat non-utilization. Bloor (1) regarded the abnormalities in blood lipoids in severe nephritis as the result of a retarded assimilation of fat in the blood, which he thought to be one manifestation of a general metabolic disturbance.

We have approached the subject by an attempt to discover whether the heaping up of lipoids in the blood is due to a retarded rate of fat combustion, or to a retardation of its deposition from the blood into the tissues. Fat metabolism experiments were planned, both on normal individuals and on nephritics with and without high blood lipoids.

Summary of Previous Literature.

The Effect of Fat Ingestion on the Total Metabolism.—Koraen (3) and Johansson and Koraen (4) concluded that there was no increase in metabolism as a result of the ingestion of fat. Gigon (5) did not always find an increase in the total metabolism, except when large amounts of fat were given, but he found an increase in the fat metabolism. Von Voit (6) stated that when 100 per cent of the basal requirement was fed to a dog in the form of fat, the total amount of protein and fat burned remained the same after ingestion of fat as during fasting. The fat ingested merely replaced the body fat in metabolism. Magnus-Levy (7), in experiments on dogs and on man, found an increase in total metabolism as a result of fat ingestion, and furthermore showed that the degree of increase was dependent on the amount of fat ingested. Rubner (8), Staehelin (9), Bartmann (10), Murlin and Lusk (11), and Benedict and Carpenter (12) all reported an increase in total metabolism after fat ingestion.

The Effect of Fat Ingestion on the Blood Lipoids.—Cholesterol: Increases in the blood cholesterol in the dog after fat ingestion were reported by Reicher (13) and by Terroine (14). No significant changes in blood cholesterol after fat ingestion in the dog were found by Bloor (15), Knudson (16), Bang (17), and Zucker (18).

Lecithin: Increases in blood lecithin after fat ingestion in the dog were reported by Reicher (13), Bloor (15), Knudson (16), and Zucker (18).

Fat and Fatty Acids: Increases in fatty acids or in neutral fats after fat ingestion in the dog were reported by Lattes (19), Reicher (13), Terroine (14), Bloor (15), Knudson (16), Zucker (18), Bang (17), and Eckstein (20). The only reports in the literature on human subjects are those of Neumann (21) and of Neisser and Braeuning (22), who reported an increase in fatty substances in the blood after fat ingestion determined by the ultramicroscopic method; those of Cowie and Hoag (23), who corroborated the results of Bloor on dogs by feeding cream to five children and three adults; and those of Bang (17), who found only slight changes in the fat content of the blood of human subjects after fat ingestion, as compared with the striking increase in the dog.

EXPERIMENTAL.

Methods.—The basal metabolism determinations were made by the Tissot spirometer method (24). The gas analyses were made with the Henderson modification of the Haldane gas analysis apparatus (25). The protein metabolism was determined by analysis of urinary nitrogen by the Kjeldahl method. When protein was present in the urine, this was removed by heating the urine and adding trichloroacetic acid until no further precipitation occurred. The mixture was diluted to a volume, filtered, and portions of the non-protein solution used for Kjeldahl determinations. All respiratory quotients given are non-protein.

The blood lipoids were determined by colorimetric and nephelometric methods. In the earlier experiments, Figs. 1, 3, and 11, in which the lipoids are recorded as total lipoid, the older method of Bloor was used (26). In the later experiments cholesterol and fatty acids were determined on plasma and on whole blood by the recent method of Bloor, Pelkan, and Allen (27). Lecithin determinations were made according to Randles and Knudson's modification of Bell and Doisy's method for the determination of phosphorus (28).

Fat Ingestion Experiments.—The basal metabolism of each subject was determined on a day previous to the day of fat ingestion, in order to familiarize the subject with the procedure, so that the factor of nervousness might be reduced as far as possible on the day of the experiment. On the day of fat ingestion the basal metabolism was determined as usual, during 10 minute periods, and blood was taken for the determination of lipoids. A meal consisting of butter (usually 1 gm. of butter per kilo of body weight) was then given. Various methods were employed to make the butter more palatable. The butter was served either in the form of balls coated with bran, or melted. 100 to 200 cc. of water were given if

Subject.	Calories per kilo per hr.			Increase in metabolism.	Respiratory quotient.		Decrease in respiratory quotient.	Total lipoids.				
								Plasma.				
	Basal.	Maximal.	Increase.		Basal.	Minimal.		Initial.	Maximal.	Change.		Initial.
	Cal.	*Cal.*	*Cal.*	*per cent*			*per cent*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*
Normals.												
G. L.	0.95	1.04	+0.09	+9.5	0.82	0.78	0.04	820	740	−80	−9.8	
	0.97	1.07	+0.10	+10.3	0.76	0.72	0.04					175
J. P.	0.87	0.98	+0.11	+12.6	0.80	0.80	0.00	650	825	+175	+27.0	
	0.94	1.03	+0.09	+9.6	0.77	0.76	0.01					210
C. L.	0.83	0.89	+0.06	+7.2	0.81	0.75	0.06					155
J. N.	1.06	1.06	0.00	0.0	0.76	0.70	0.06					160
R. B.	0.84	0.97	+0.13	+15.5	0.80	0.73	0.07					195
H. S.	1.02	1.06	+0.04	+3.9	0.76	0.76	0.00					195
Average				+8.5			0.035				+8.6	
Nephritics with normal blood lipoids.												
H. L.	1.08	1.19	+0.11	+10.2	0.82	0.72	0.10					180
J. O'M.	1.25	1.40	+0.15	+12.0	0.81	0.77	0.04					240
Average				+11.1			0.07					
Nephritics with high blood lipoids.												
F. R.	0.94	1.00	+0.06	+6.4	0.88	0.80	0.08	990	1,070	+80	+8.1	
J. D.	1.20	1.26	+0.06	+5.0	0.83	0.78	0.05					410
	1.16	1.39	+0.23	+19.8	0.87	0.81	0.06					250
M. F.	0.94	1.08	+0.14	+14.9	0.85	0.75	0.10					460
L. S.	0.89	0.96	+0.07	+7.9	0.78	0.75	0.03					620
	0.92	1.03	+0.11	+12.0	0.83	0.75	0.08					510
B. S.	1.26	1.39	+0.13	+10.3	0.82	0.75	0.07					580
Average				+10.9			0.067					

TABLE I.

olic and Blood Lipoid Changes after Fat Ingestion in Normal and in Nephritic Subjects.

Changes in lipoid content per 100 cc. of plasm

Total lipoids.			Cholesterol.								
Plasma.			Plasma.				Whole blood.				
ximal.	Change.		Initial.	Maxi-mal.	Change.		Initial.	Maxi-mal.	Change.		Initial.
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*
740	−80	−9.8									
			175	175	0	0.0					
825	+175	+27.0									
			210	230	+20	+9.5					
			155	175	+20	+12.9					
			160	160	0	0.0	150	150	0	0.0	190
			195	195	0	0.0	135	145	+10	+7.4	250
			195	210	+15	+7.7	180	165	−15	−8.3	220
		+8.6				+5.0				−0.3	
			180	195	+15	+8.3					
			240	240	0	0.0					
						+4.1					
070	+80	+8.1									
			410	450	+40	+9.9					
			250	290	+40	+16.0					
			460	460	0	0.0	355	349	−6	−1.7	425
			620	730	+110	+17.7					
			510	485	−25	−4.9					
			580	570	−10	−1.7	380	375	−5	−1.3	430
						+6.1				−1.5	

ma and whole blood.

Lecithin.							Fatty acids.			
Plasma.			Whole blood.				Plasma.			
Maximal.	Change.		Initial.	Maximal.	Change.		Initial.	Maximal.	Change.	
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*
							325	265	−60	−18.5
							355	640	+285	+80.0
							190	245	+55	+29.0
205	+15	+7.9	275	285	+10	+3.6	380	385	+5	+1.3
250	0	0.0	300	300	0	0.0	545	640	+95	+17.4
220	0	0.0	295	295	0	0.0	360	440	+80	+22.2
		+2.6				+1.2				+21.9
							450	470	+20	+4.5
							550	550	0	0.0
										+2.2
							1,040	1,270	+230	+22.1
							580	1,000	+420	+72.4
465	+40	+9.4	470	520	+50	+10.6	750	1,220	+470	+62.7
							965	1,105	+140	+14.5
							980	1,250	+270	+27.6
510	+80	+18.6	415	465	+50	+12.0	950	1,620	+670	+70.5
		+14.0				+11.3				+45.0

desired. Water ingested in such small amounts has no effect on the metabolism (29). Bran was used because it contains no material which is absorbed, and, therefore, does not enter into the metabolism. Intestinal activity alone does not enter into the changes in basal metabolism (30). Rubner (8) showed that a dog could eat bones without increasing his metabolism. The metabolism was determined by 10 minute periods, 1, 3, and 5 hours after the fat ingestion. Blood and urine samples were taken immediately following the initial basal metabolism, and the 3 and 5 hour metabolism periods. The subject was kept as quiet as possible during the entire period of the experiment in order to eliminate as far as possible all factors affecting the metabolism. No determinations of fat in the feces were made.

Control experiments were performed on six normal individuals. The results of these controls are shown in Figs. 1 to 8. Seven patients with nephritis of various types were examined. Two of these cases were patients with normal blood lipoids. One, shown in Fig. 9, was a case of nephrosclerosis with no signs of renal insufficiency. The other, Fig. 10, was a case of subchronic glomerulonephritis with marked renal involvement. Five of these patients, Figs. 11 to 19, were cases with increased blood lipoids. Three were suffering from nephrosis: L.S., B.S., and F.R., Figs. 16, 17, 18, 19, and 11; but the last of these was in a state of almost complete recovery. The remaining two patients, J.D., and M.F., Figs. 12, 13, 14, and 15, were cases of chronic glomerulonephritis. Control experiments were performed on two of these patients with high blood lipoids, Figs. 15 and 19. The procedure was the same as in the fat ingestion experiments, except that no fat was given, and the experiment was carried on only to the 3rd hour. We did not wish to fast the patient longer than necessary, and after the 3rd hour the significant increases had already occurred in the majority of experiments. In all cases in which edema existed, the basal metabolism was calculated on the patient's weight at the time of disappearance of the edema, when the weight became constant.

Table I gives a summary of all the experiments, with averages of the results. Table II gives the character of the diet of the patient, and the amount of fat given on the day of the experiment.

TABLE II.

Diet Previous to and on Day of Fat Ingestion.

Subject.	Body weight.*	Diet 10 days prior to fat metabolism experiment.					Basal requirement.		Amount of fat ingested.	Fat ingested in per cent of 6 hr. requirement.	Date of fat feeding.	Basal metabolic rate, deviation from Du Bois' average normal.†
		Total calories.	Per cent of basal requirement.	Calories per kilo.	Protein.	Fat.	24 hr.	6 hr.				
	kg.	*cal.*	*per cent*	*cal.*	*gm.*	*gm.*	*cal.*	*cal.*	*cal.*	*per cent*		
Normals.												
G. L.	66.0						1,550	387.5	460	118.6	Sept. 28, 1922	−11.4
J. P.	77.5						1,740	435.0	460	105.6	Oct. 3, 1922	−5.9
C. L.	90.4						1,800	450.0	460	102.2	" 10, 1922	−7.9
J. N.	58.0						1,490	372.5	446	112.7	May 2, 1923	−8.0
R. B.	77.0						1,600	400.0	460	115.0	Apr. 16, 1923	−10.8
H. S.	72.1						1,750	437.5	514	117.5	Mar. 26, 1923	−3.7

Nephritics.												
H. L.	51.1	1,850	137	36.2	55	60	1,350	337.5	383	113.5	Dec. 11, 1922	+3.0
J. O'M.	40.7	1,650	133	40.6	55	60	1,240	310.0	307	99.0	" 18, 1922	−18.3
F. R.	60.2	2,000	148	33.3	70	50	1,350	337.5	460	136.3	May 25, 1922	−22.3
J. D.	36.0	1,600	146	44.4	60	70	1,100	275.0	307	111.6	June 8, 1922	−25.6
	36.0	1,700	155	47.3	60	65	1,100	275.0	307	111.6	Oct. 17, 1922	−28.3
M. F.	48.0	1,700	155	35.4	50	70	1,100	275.0	345	125.5	Apr. 23, 1923	−19.0
L. S.	62.5	1,000	77	16.0	35	45	1,300	325.0	383	118.0	Oct. 30, 1922	−19.2
	62.5	1,600	123	25.6	70	55	1,300	325.0	383	118.0	Dec. 4, 1922	−18.7
B. S.	33.9	1,500	125	44.3	40	50	1,200	300.0	307	102.3	Apr. 3, 1923	−20.7
		1,600	139	47.2	70	30	1,150	287.5			" 26, 1923	−20.0

* Body weights recorded are weights after the disappearance of edema.

† Normal subjects show deviations up to ± 15 per cent from the average.

DISCUSSION.

Basal Metabolic Rate.—The basal metabolic rate of the normals varied from −3.7 to −11.4 per cent from Du Bois' average normal standard, averaging −7.9 per cent (Table II). A few of these rates are slightly low according to the older standards of ±10 per cent, but all fall within the normal range as extended by Boothby (31) in his recent report of 8,614 determinations of basal metabolic rate. Of these, 127 were normals. He has concluded that although the great majority of normals fall within the ±10 per cent range, a sufficient number extend to a ±15 per cent range to make it necessary to extend the standard for normals to that zone, using the Du Bois standards for comparison. The hypertension case, H.L., Fig. 9 and Table II, had a normal rate of +3 per cent. All of the nephritic cases, Figs. 10 to 19 and Table II, had a lowered metabolic rate, ranging from −18.3 to −28.3 per cent. Whether the lowered metabolic rate found in all of our nephritics is a manifestation of the disease or not, we are not prepared to say. We think that the low metabolic rate may be due to the fact that all of our patients had been on a low caloric diet for some time, which is capable of depressing the rate of metabolism, as shown by Benedict in his reports on inanition and reduced diet (32). Our patients were all kept well above their basal requirement, as shown in Table II. The diets averaged 146 per cent of the 24 hour basal requirements, but this is low when compared to the ration of a normal individual who is up and about. Furthermore, most of our patients had been inactive and in bed for several months before the determination was made.

Effect of Fat Ingestion on Metabolism.—The metabolism of the normals after fat ingestion showed some variations, as shown in Figs. 1 to 8, and in Tables I and II. The increment in metabolism varied from 0 in one case, to 15.5 per cent, with an average of 8.5 per cent. This increase agrees fairly well with the results previously reported in the literature (3–12). The majority of investigators fed much larger quantities of fat than were used in our experiments, which accounts for their greater increment in the metabolism, as shown by Magnus-Levy (7). The amount of fat which our subjects ate varied between 99 and 136 per cent of the basal requirement for the 6 hours of the experiment,

as shown in Table II. The increase in metabolism in the nephritic cases varied from 5 to 19.8 per cent, averaging 10.9 per cent (Table I). There was no difference in the effect on total metabolism between normals and nephritics in their reaction toward fat ingestion.

Changes in Respiratory Quotient.—The respiratory quotients in two normals showed no significant change throughout the experiment. The remaining four showed a decided drop (Table I) which signified that fat was being burned to a greater extent after the fat ingestion than before. The nephritic cases showed the same decrease in respiratory quotient after fat ingestion, so that there was no marked difference in the degree and the kind of metabolism between normals and nephritics. That these changes are not due to natural variations in the course of the day is shown by control experiments on two patients, Figs. 15 and 19, in which there was no change in respiratory quotient and no increase in metabolism.

Effect of Fat Ingestion on Blood Lipoids.

Cholesterol.—The blood cholesterol curves are for the most part straight lines. The average increase in plasma cholesterol for normals was 5 per cent, and for nephritics 6.1 per cent (Table I). The changes in the cholesterol of whole blood averaged −0.3 per cent for normals and −1.5 per cent for nephritics. The changes are very slight, and nearly all fall within the limits of experimental error. The cholesterol picture does not agree with the earlier reports of Reicher (13) and Terroine (14), who found an increase in the cholesterol, but agrees with later reports by Bloor (15), Knudson (16), Bang (17), and Zucker (18), who found the blood cholesterol unchanged after fat ingestion, using the dog as a subject.

Lecithin.—The lecithin of both plasma and whole blood showed no change after fat ingestion in two normals, Figs. 7 and 8, and Table I. In one normal, Fig. 5 and Table I, the lecithin increased 7.9 per cent in the plasma and 3.6 per cent in the whole blood. These results do not corroborate those obtained when dogs were used as subjects. Reicher (13), Bloor (15), Knudson (16), Zucker (18), and Eckstein (20), all report an increase in blood lecithin during fat absorption in the dog. Different species do not always react in the same way, and

different animals in the same species do not always show the same reactions toward lipoids, as shown by Bang (17), by Neisser and Braeuning (22), and by Bloor (15). All lecithin observations in the literature were made on dogs. Our average increase in lecithin in three normals is 2.6 per cent for plasma, and 1.2 per cent for whole blood.

The nephritic cases with high lipoids showed greater increases than the normals. Determinations were made on two nephritics, M.F. and B.S., Figs. 14 and 18, and Table I. The average increase in lecithin was 14 per cent for plasma and 11.3 per cent for whole blood. Control experiments were performed on both of these patients, which showed no change in the lecithin of plasma or whole blood during a similar period when no fat was ingested.

Fatty Acids.—The increase in the fatty acids of the blood of normals after fat ingestion is slight. Of the six subjects used, one showed a decrease in the fatty acids of the plasma, four showed an increase varying from 1.3 to 29 per cent, and one showed an increase of 80 per cent (Table I). The latter subject, J.P., Figs. 3 and 4, was the only normal who showed any considerable increase in fatty acids. Similar results were obtained in two different experiments. In the first experiment, Fig. 3, the total lipoids were determined (26). In the second experiment, Fig. 4, fatty acids and cholesterol were determined separately (27), and the fatty acids present a curve almost as steep as some of the severe nephritic curves. The average increase in plasma fatty acids was 21.9 per cent. These results, which we have obtained on human subjects, with the one exception of J. P., are very different from the results obtained on dogs by Lattes (19), Reicher (13), Terroine (14), Bloor (15), Knudson (16), Zucker (18), Eckstein (20), and Bang (17), all of whom report a larger increment, varying between 50 and 245 per cent. Our results, however, agree very well with those reported by Bang (17) in his experiments on human subjects. He fed fat in the form of butter and cream, amounting to 150 gm. of fat. Of the twelve subjects, three showed no increase $1\frac{1}{2}$ hours after ingestion, three showed an increase of less than 20 per cent, the remaining six showed an increase varying between 28 and 67 per cent. The average increment was 28.4 per cent. The average increment for our six normals was 21.9 per cent (Table I). There are two possible reasons why our

results in the fatty acids should differ from the observations made on dogs, aside from the greater factor of difference in species. In the first place, in the majority of experiments performed on dogs, the fat was ingested in the form of olive oil (Bloor (15), Bang (17), and Zucker (18)). Bang (17) showed that when lard was fed to dogs no hyperlipemia appeared, when butter was fed there was often a hyperlipemia, but when olive oil was used, a hyperlipemia always occurred. He also found great variations in individual reactions of dogs toward ingested fat. Neisser and Braeuning (22) also stated that the amount of increase in blood lipoids varied with the kind of fat fed, and with the animal. If the same variations due to the kind of fat ingested hold good for men as well as for dogs, the fact that the dogs were usually fed on olive oil and our subjects were fed on butter would account for some of the differences in results. The variations found by Bang (17) in individual reactions in dogs undoubtedly also hold good in the case of men, as shown by the great variations exhibited in Bang's figures for men (17) and also by our case of J. P., Figs. 3 and 4, and Table I. In the second place, our subjects received much smaller amounts of fat than any of the animals reported. Reicher's dogs (13) received 5 or 6 gm. of fat per kilo of body weight; Bloor's dogs (15) received from 7 to 16 gm. per kilo; Knudson's (16) and Zucker's (18) about 5 gm. per kilo, and Bang's (17) about 3 to 5 gm. per kilo. Our subjects received about 1 gm. of butter per kilo, which is somewhat less than 1 gm. of fat per kilo. The dosage, therefore, averages about one-fifth of that given in the dog experiments, so one would expect less of an increase in the blood lipoids. Bloor (15) stated that the increased amount of fat in the blood might be due to the introduction of a quantity of fat greater than the temporary storehouse can take care of, with resulting accumulation. It is probable that the amount of fat which our human subjects received was within the limits of the amount which the average normal can dispose of, and that amount is probably removed from the blood as rapidly as it is taken up by it.

Two patients, H. L., nephrosclerosis, and J. O'M., subchronic glomerulonephritis, who showed normal blood lipoids, Figs. 9 and 10, and Table I, showed an increase in fatty acids of 4.5 and 0 per cent respectively, which comes well within our range for normals. The five patients with high blood lipoids, Figs. 11 to 19, and Table I,

however, showed much greater increases in plasma fatty acids than did the normals. The increase varied from 14.5 to 72.4 per cent, averaging 45 per cent. Since the normals averaged 21.9 per cent, the patients with high blood lipoids showed twice as great an increment as did the normals. Control experiments were performed, Figs. 15 and 19, to show that there was no significant change in blood lipoids in the course of the experiment when no fat was ingested.

Since the patients received approximately the same amount of fat per kilo of body weight as the normals, and yet showed a much greater increment in fatty acids and lecithin in the blood, the difference may lie in the ability of the organism to remove lipoids from the blood. The normal organism evidently has some mechanism, perhaps having some connection with the transport to and temporary storage in the liver, as suggested by Leathes (33), by which certain amounts of fat ingested are removed from the blood with a rapidity almost equal to that of their entrance into the blood. The increase in blood lipoids found in cases of nephritis may be due to some disturbance of this balancing mechanism, whereby the lipoids are much more slowly removed from the blood than under normal conditions. This disturbance apparently has no effect on the ability of the organism to burn fat, since the nephritic patients with high blood lipoids showed the same decrease in respiratory quotient as did the normals, which indicated that they had an equal ability to burn fats.

SUMMARY.

Determinations of the plasma lipoids and of the respiratory quotient and total metabolism (Tissot method) have been performed with nephritics and normal subjects before and after they ingested fat in the proportion of 1 gm. per kilo body weight.

After fat ingestion a greater increase of fatty acids and lecithin was noted in the plasma of nephritics with initially high blood lipoids than in the plasma of normal subjects or of nephritics without constant lipemia. In cholesterol no differences were found.

The nephritic patients with constant lipemia were able to burn fat as efficiently as normal individuals.

The accumulation of fat in their blood may be due to a disturbance in the mechanism for transferring lipoids from the blood to the tissue depots.

Explanation of Figures.

The abscissæ represent the number of hours after fat ingestion. The ordinates have a single scale which is used for all curves: For the metabolism curve, the ordinates represent calories per kilo per hour. For the lipoid curves the ordinates represent grams per 100 cc. of plasma or blood. The following signs are used to indicate the different curves.

●——● Calories per kilo per hour.
+——+ Non-protein respiratory quotient.
□ – – □ Whole blood fatty acids—per cent.
□——□ Plasma fatty acids—per cent.
△ – – △ Whole blood lecithin—per cent.
△——△ Plasma lecithin—per cent.
○ – – ○ Whole blood cholesterol—per cent.
○——○ Plasma cholesterol—per cent.
⊙——⊙ Total plasma lipoids—per cent.

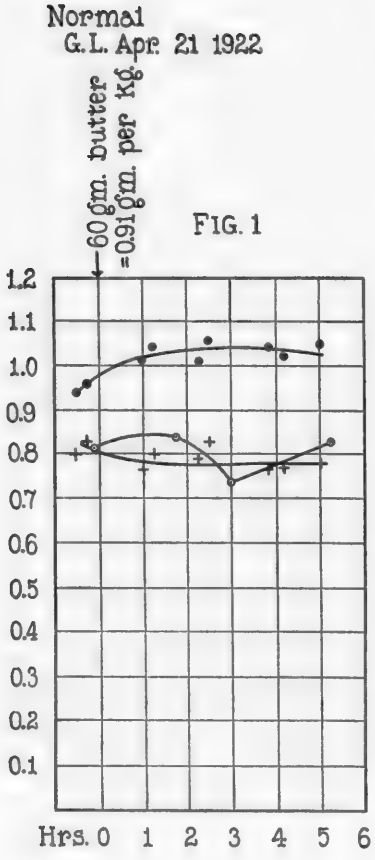

Normal
G.L. Apr. 21 1922
60 gm. butter
=0.91 gm. per kg.
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6

FIG. 1

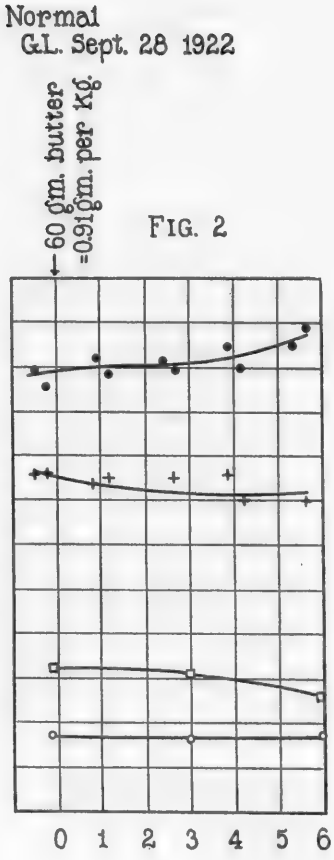

Normal
G.L. Sept. 28 1922
60 gm. butter
=0.91 gm. per kg.
0 1 2 3 4 5 6

FIG. 2

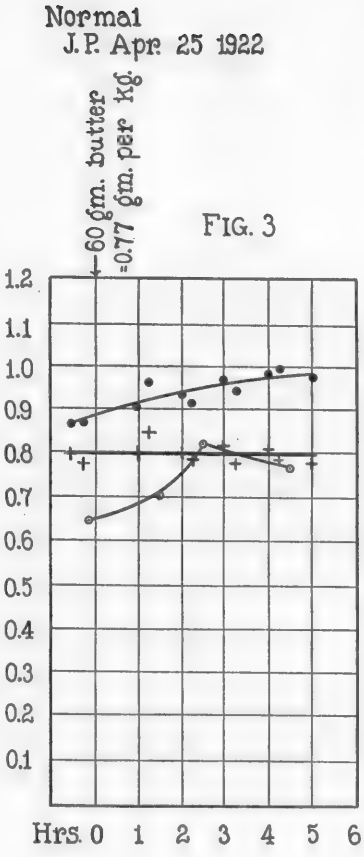

Normal
J.P. Apr. 25 1922
60 gm. butter
=0.77 gm. per kg.
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6

FIG. 3

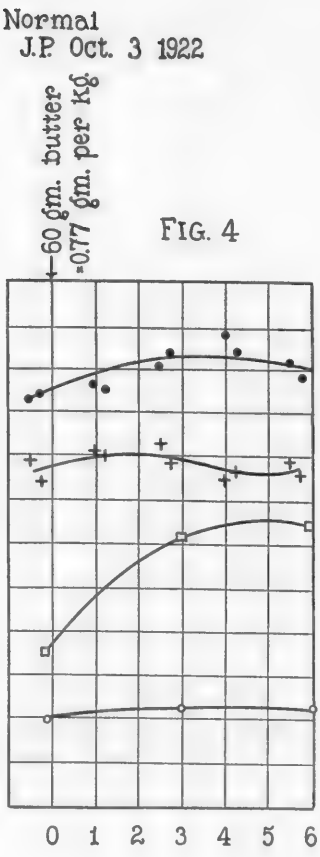
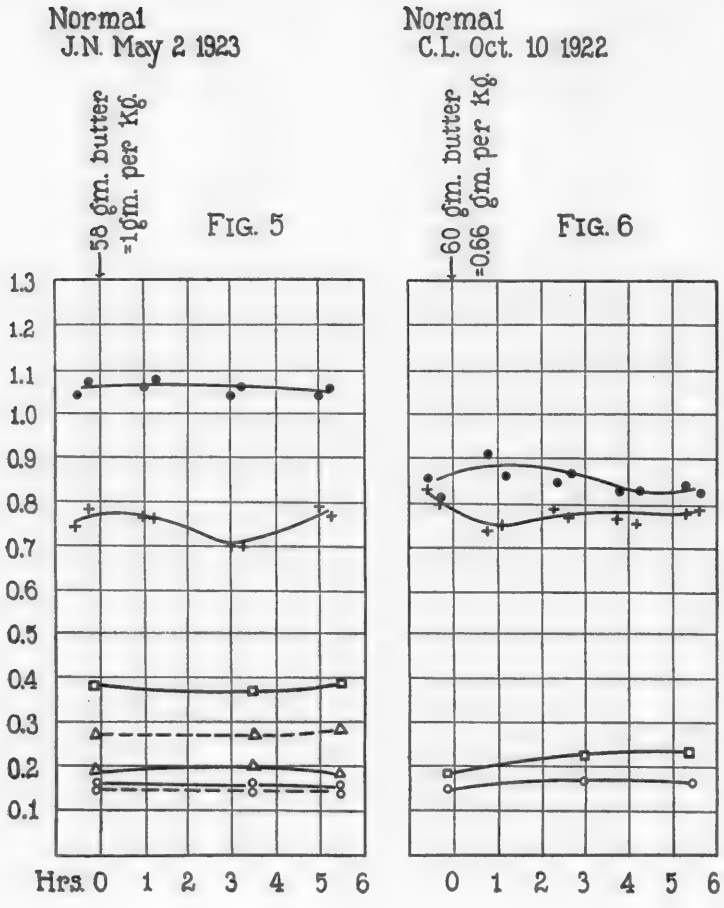

Normal
J.P. Oct. 3 1922
60 gm. butter
=0.77 gm. per kg.
0 1 2 3 4 5 6

FIG. 4


Normal
J.N. May 2 1923
58 gm. butter
=1 gm. per kg.
FIG. 5
Normal
C.L. Oct. 10 1922
60 gm. butter
=0.66 gm. per kg.
FIG. 6
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6
0 1 2 3 4 5 6

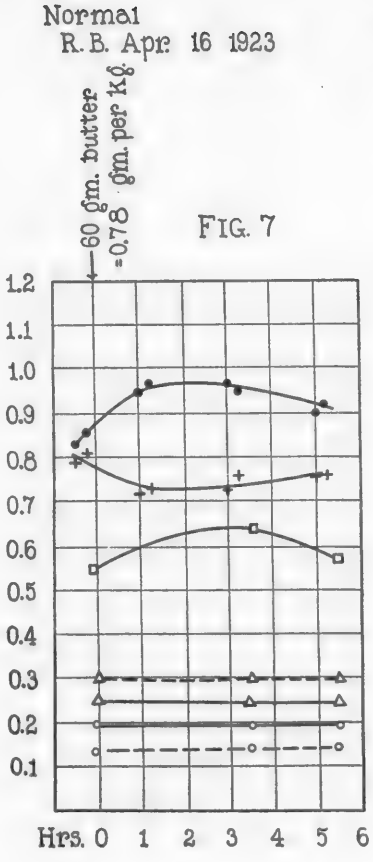

Normal
R.B. Apr. 16 1923
60 gm. butter
=0.78 gm. per kg.
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6

FIG. 7

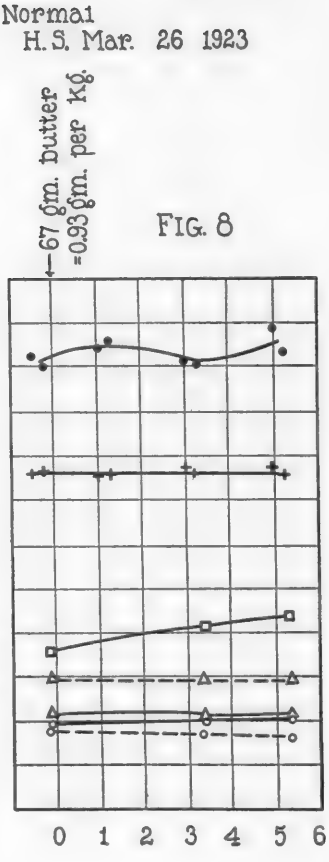

Normal
H.S. Mar. 26 1923
67 gm. butter
=0.93 gm. per kg.
0 1 2 3 4 5 6

FIG. 8

Nephritic with normal blood lipoids
H.L. Dec. 11 1922

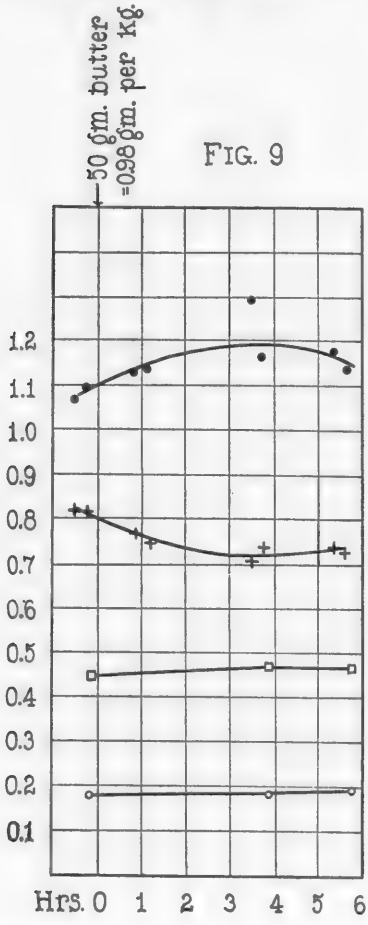


FIG. 9

Nephritic with normal blood lipoids
J.O'M. Dec. 18 1922

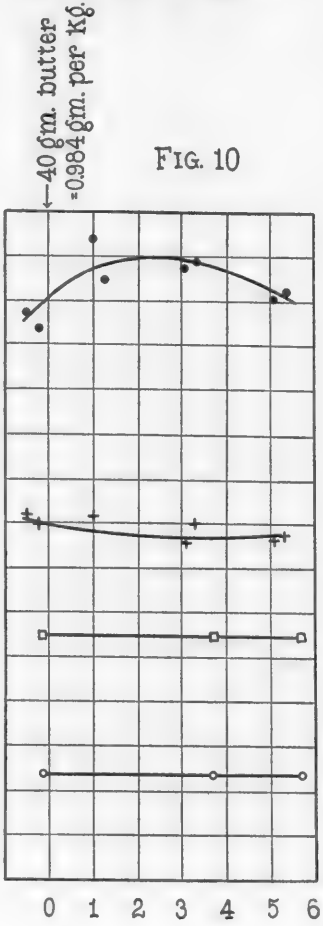


FIG. 10

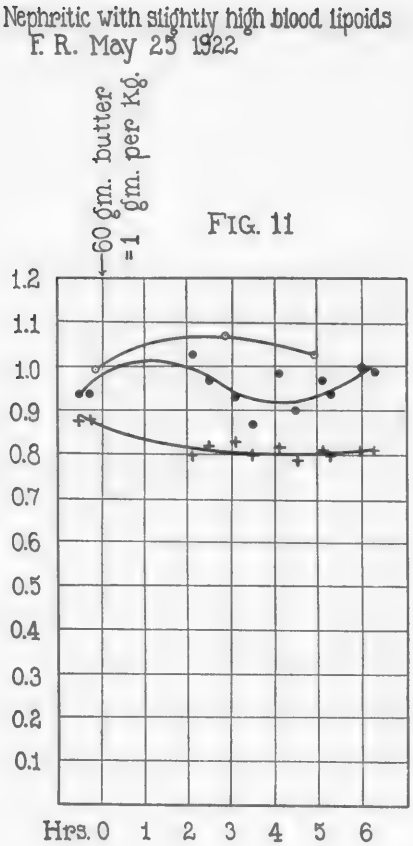

Nephritic with slightly high blood lipoids
F. R. May 25 1922
60 gm. butter
= 1 gm. per Kg.
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6

FIG. 11

Nephritic with high blood lipoids
J. D. June 8 1922

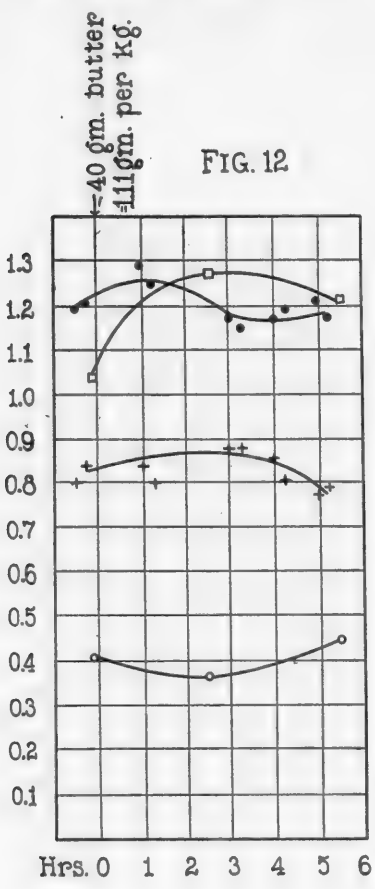


FIG. 12

Nephritic with high blood lipoids
Oct. 17 1922

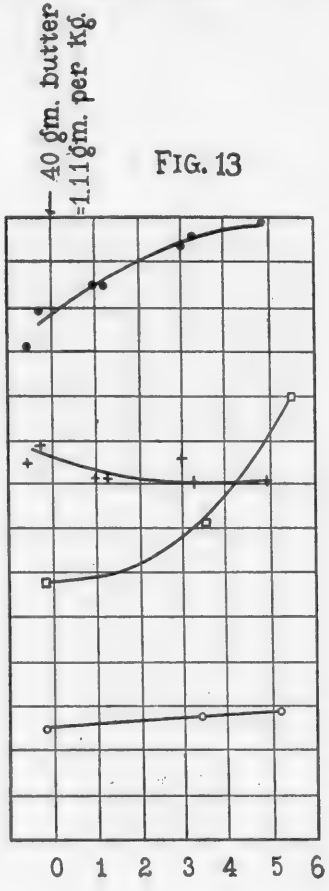


FIG. 13

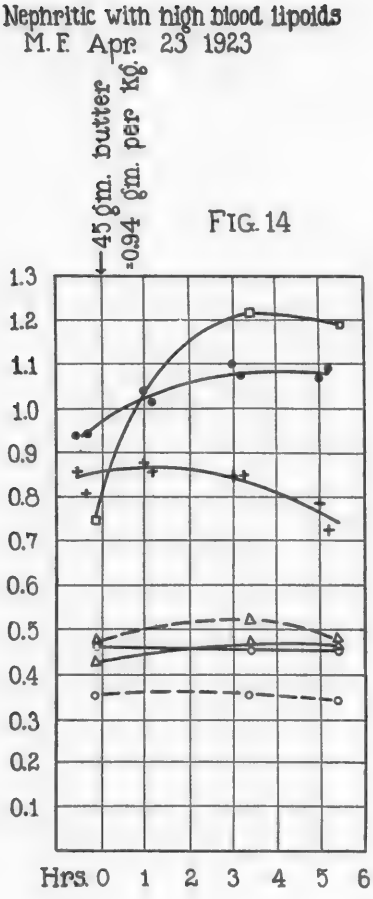

Nephritic with high blood lipoids
M. F. Apr. 23 1923
45 gm. butter
=0.94 gm. per kg.
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Hrs. 0 1 2 3 4 5 6

FIG. 14

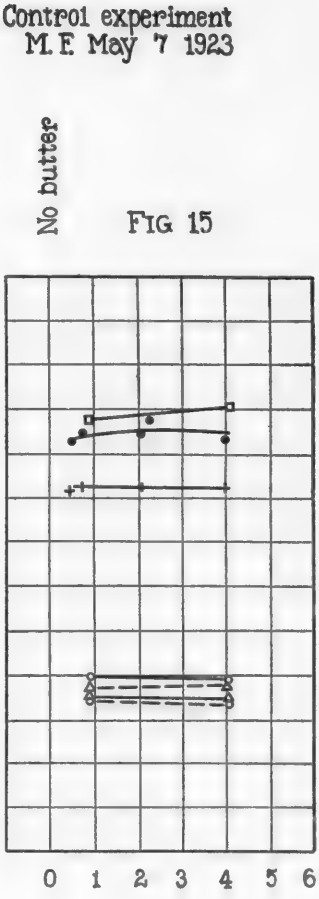

Control experiment
M. F. May 7 1923
No butter
0 1 2 3 4 5 6

FIG. 15

Nephritic with high blood lipoids
L. S. Oct. 30 1922

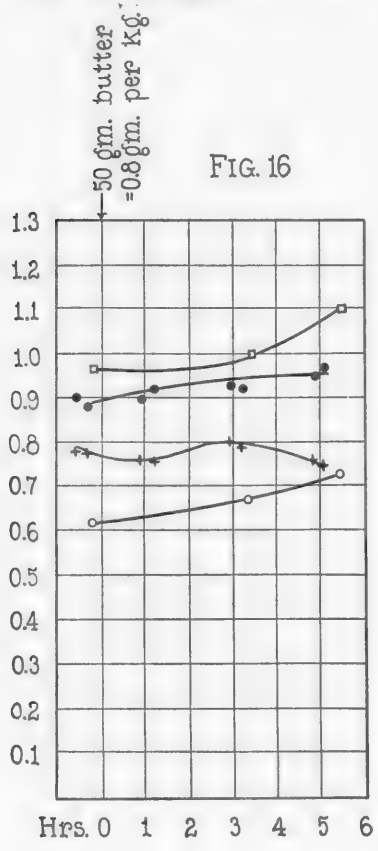


FIG. 16

Nephritic with high blood lipoids
L. S. Dec. 4 1922

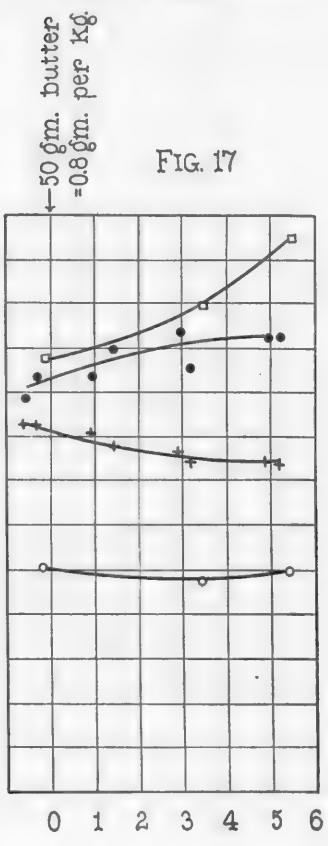


FIG. 17

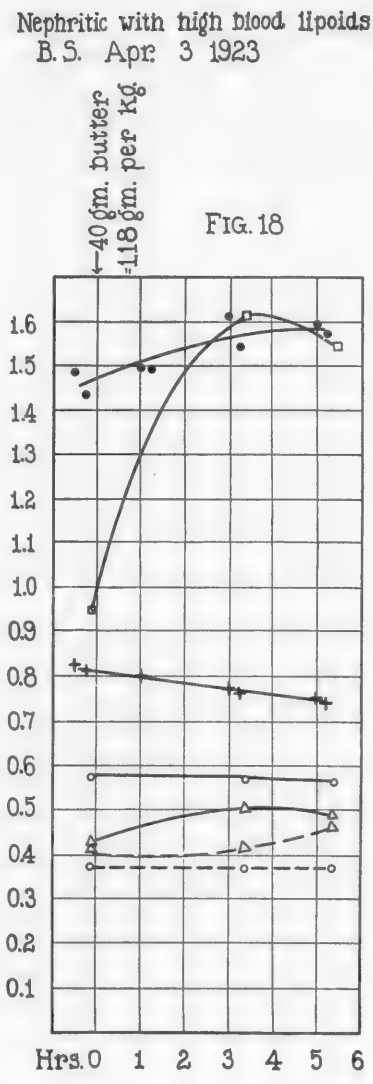


FIG. 18

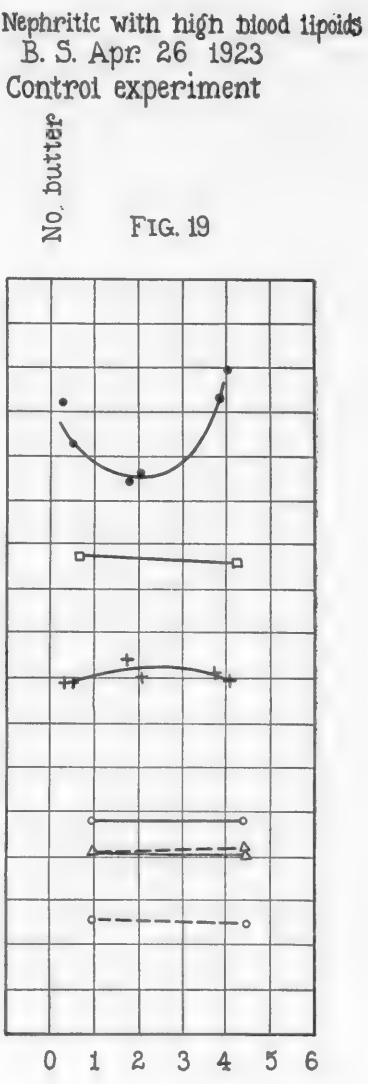


FIG. 19

At the time of the metabolism represented by the 1st hour in Fig. 19, the patient was very drowsy, and was almost asleep, which accounts for the dip in the curve at this point.

PROTOCOLS.

L. S.—Case of acute nephrosis. Male, 30 years. In September, 1922, following gastroenteritis, he became edematous and a considerable proteinuria was found. Investigation revealed a blood urea nitrogen of 0.12 gm. per liter, a plasma cholesterol of 5.50 gm. per liter, a phthalein output of 30 per cent in 2 hours, and a basal metabolic rate of +1 per cent. A diagnosis of nephrosis was made and a high protein diet prescribed. 2 weeks later the edema had greatly increased and become generalized, and there were occasional nausea and vomiting. The blood urea nitrogen was 0.39, the plasma cholesterol 4.6, and the basal metabolism −9.3. Thyroid was given without benefit. The urine progressively diminished in amount. There were no headaches, and no visual disturbances, but the vomiting increased and there was slight dyspnea.

On admission, October 6, 1922, he had great anasarca with hydrothorax and ascites. No retinal changes were present. Blood pressure 120/80; no cardiac hypertrophy; arteries healthy. The urine contained from 9 to 17 gm. of protein per liter; specific gravity 1,040; the deposit contained many casts, leucocytes, and a few blood cells. The blood urea nitrogen was 0.47 and urine urea nitrogen 13.0 gm. per liter. The plasma proteins were much depleted and the albumin-globulin ratio inverted. Wassermann reaction negative. Anemia slight.

A milk diet was given until the gastric symptoms had subsided, when bread, butter, and cereals were added. 30 gm. of urea were given daily and caused a slight increase in the output of urine without increasing the blood urea. On October 28, 2,600 cc. of fluid were aspirated from the right pleural sac. Improvement was fitful and by the end of October little progress had been made. At this time the first fat metabolism experiment was made.

From November 4 a diet containing 20 gm. of fat, 250 gm. of carbohydrate, and 60 gm. of protein was given, the protein being increased to 70 gm. on the 17th. The slow improvement continued unaltered until the 14th when a progressive increase in the output of water and chlorides began; this was followed by a dramatic fall in the blood urea to 0.17 on the 19th, while the urea feeding continued, and in body weight which reached 62 kilos on December 6. Plasma cholesterol on November 19 was 5.1 gm., and on November 24, 7.9 gm. By December 1 no edema remained and no fluid was present in the chest or abdomen. On November 24 in the middle of the diuresis the fat in the diet was increased to 40 gm. and the protein to 85 gm. without any change in the rate of improvement. On December 4 the second fat metabolism experiment was performed.

The subsequent history was uneventful and ended in an apparently complete recovery.

B. S.—Case of chronic nephrosis. Patient was a boy, 11 years old, who had had edema of the face and legs, and proteinuria since February, 1922. No cause was known. He felt perfectly well.

Admitted in November, 1922, he had a puffy face, and slight pitting of the right leg; the urine contained 4 gm. of protein per liter; the sediment was normal. The

plasma proteins were diminished, and the albumin-globulin ratio inverted. There were no vascular changes. Renal function tests elicited normal responses. The blood urea nitrogen was 0.07 gm. per liter. The edema disappeared, and he was discharged in December with a trace of protein in the urine.

In March he was readmitted with bilateral pleural effusion, ascites, and edema of the face and legs. Weight 40.6 kilos. On April 3 he was still in this condition; weight 39.5 kilos. Thyroid extract was given at the end of April, and was followed by improvement. On May 15 there were no signs of fluid and the weight was 34.5 kilos.

F. R.—Case of acute nephrosis. Female, 20 years. She was admitted in November, 1921, with moderate edema, proteinuria, a normal blood pressure, and normal blood urea. The plasma cholesterol was 7.9 gm. per liter, and the total fats 20 gm. per liter (old Bloor method). She developed a series of erythemata of obscure infective nature, and the edema greatly increased. In January and February the edema subsided while she was taking a high protein, low fat diet. In May, when the fat metabolism experiment was performed, she was convalescent; response to function tests was normal; the blood pressure was normal; and the urinary protein was reduced to a trace.

M. F.—Case of subacute glomerulonephritis. Female, 25 years old. Her ankles and legs became edematous in October, 1922. She remained at work until December, but the swelling steadily increased, and spread to the trunk and arms. Her vision became affected, but there were no symptoms of uremia.

Admitted January 4, 1923, she showed massive edema of the legs and lower part of the trunk, and moderate edema of the arms, face, and chest wall. Albuminuric retinitis was present in both eyes. Heart was not hypertrophied, but the blood pressure was 205/128. The pleuræ contained fluid. Urine: smoky, 3.5 gm. protein per liter, the deposit containing many casts and blood cells. Blood urea nitrogen 0.46 gm. per liter. Red blood corpuscles 3,520,000 and hemoglobin 62 per cent. Wassermann reaction negative.

On a milk diet and with daily sweat baths the edema diminished but the blood urea nitrogen rose to 0.80, and uremic symptoms developed. At the end of January she began to improve, her weight, blood urea, and blood pressure falling together, and by the middle of February she had no edema, no pleural effusion, and a blood pressure of 160/102. The urine remained smoky until March, and in May still contained numerous red blood corpuscles and casts. The blood urea nitrogen in April was 0.55 and the phthalein output 26 per cent in 2 hours. The red blood corpuscles had fallen to 2,200,000 and the hemoglobin to 47 per cent. She remained free from edema.

Weight on admission 65.8 kilos.

Weight after loss of edema 48 kilos.

J. D.—Case of subchronic glomerulonephritis. The patient, a boy of 14 years, developed edema in May, 1922, without any preceding illness or infection.

On admission there was marked general edema with effusions into the pleura and peritoneum. The area of cardiac dullness was enlarged, and the blood pres-

sure 135/90. The urine contained 4 gm. of protein per liter, a large amount of blood, and many casts. The blood urea nitrogen was 0.2 gm. per liter, and the phthalein output 46 per cent in 2 hours. Red blood corpuscles 4,100,000, and hemoglobin 65 per cent.

The edema rapidly subsided, and his weight fell from 50 kilos on May 19, to 38 kilos on June 9. By the beginning of June there were no signs of fluid in the chest or abdomen, and slight pretibial edema alone remained. The hematuria was persistent. He developed an increasing anemia, the red blood corpuscles being 2,400,000 in March, 1923. In February definite pathological changes were noted in the fundi. No nitrogen retention developed and his blood pressure fell to 120/70 soon after admission and did not rise again. The edema did not recur, although slight pretibial pitting persisted.

J. O'M.—Case of subchronic glomerulonephritis. The patient was a boy of 14 years. In September, 1922, his illness began with edema of the ankles; his urine contained albumin and blood. There was no history of an infection.

On admission on October 8 there was edema of moderate degree, the blood pressure was 116/70, and the area of cardiac dullness slightly increased. The urine contained 2 gm. of protein per liter, much blood, and many casts. The blood urea nitrogen was 0.4 gm. per liter. Wassermann reaction negative. Red blood corpuscles 3,800,000. Hemoglobin 55 per cent.

The edema rapidly disappeared, his weight falling from 47 kilos to 41 in 7 days. The blood urea nitrogen fell very slowly, being 0.26 gm. in January, 1923, and 0.19 in March. The anemia persisted. Renal function tests showed marked impairment. The urine continued to show protein and casts in large quantities, but the hematuria diminished considerably. The blood pressure did not rise, and no retinal lesions developed. He remained free from edema.

H. L.—Case of nephrosclerosis. The patient, a nervous woman of 29 years, had been ailing since March, 1922. In September raised blood pressure was discovered.

Admitted in November she presented a blood pressure of 215/130 without recognizable cardiac hypertrophy or arterial degeneration. There was a trace of protein in the urine. There were no retinal changes, no edema, and nothing abnormal in the urinary sediment. The blood urea nitrogen was 0.15 gm. per liter, and renal function tests (phthalein, iodide, urea feeding, dilution, and concentration) revealed no impairment of renal function. No focus of infection was found. Wassermann reaction negative. A salt-free diet with moderate amount of protein was given, and she received hot air baths and iodide medication. By the middle of December her blood pressure had fallen to 170.

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